Clavicipitacean Fungi
Evolutionary Biology, Chemistry, Biocontrol, and Cultural Impacts

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The clavicipitalean fungi comprise all sexual and asexual relatives that fall within the phylogenetically defined ascomycete family Clavicipitaceae. The family includes well-known genera *Balansia*, *Beauveria*, *Claviceps*, *Cordyceps*, *Ephelis*, *Epichloë*, *Hypocrella*, *Metarhizium*, and *Neotyphodium*, as well as some lesser-known genera, such as *Hyperdermium*, *Munkia*, *Myriogenospora*, *Paeclomyces*, *Tolypocladium*, and *Ustilaginoidea*. Clavicipitaleans have specialized as insect, plant, and fungal pathogens, with many effects on hosts, their ecology, and humans.

One clavicipitalean, the ergot fungus (*Claviceps purpurea*), caused tremendous human suffering as a contaminant of cereal crops, resulting in countless historical outbreaks of ergotism in humans. Consumption of grains contaminated with hallucinogenic alkaloids of this fungus are hypothesized to have played a role in causing bizarre behaviors that triggered witch trials in Salem, Massachusetts, and Europe centuries ago. The same fungus was the source of the psychedelic drug LSD, which spawned the “psychedelic era” of the 1960s.

Another group of clavicipitaleans is the well-known “grass endophytes” (*Epichloë/Neotyphodium*), which live inside grasses as mutualistic endosymbionts, filling grasses with their secondary metabolites, resulting in protection of
host plants from herbivory of insects and mammals, and increasing disease resistance and drought tolerance. These clavicipitalean endophytes have caused many historical and modern livestock losses due to toxins that they produce. Several additional species of clavicipitaleans are important as sources of medicines and biological control agents.

Because of the importance of and potential for discovery of future applications of this group of fungi, there is value in taking a holistic view of the entire family. This may enable the identification of common biological, biochemical, and genetic features and permit generalizations that might not otherwise develop. This book is an attempt to combine studies of applications, diversity, ecology, evolution, genetics, physiology, and taxonomy of a diverse range of clavicipitaleans into a single volume that will enable others to develop knowledge of the entire family.

We confess that, as important a contribution to knowledge of the fungi as this book makes, much remains to be done. We must develop a better understanding of the evolutionary development of the group on a genomic and a biochemical level. We need a better understanding of the mechanisms of parasitism and biotrophy of hosts. We are hopeful that this volume will provide a foundation for future students of these fungi.

The editors are grateful to Ms. Elizabeth Lewis for her contributions in assisting the editing of this book.

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Historical Perspectives: Human Interactions with Clavicipitalean Fungi

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1. INTRODUCTION

The clavicipitaleans have a long, bittersweet relationship with humans. They have been sources of human suffering while also lightening human burdens in several ways. Clavicipitaleans such as Claviceps and Epichloë were the causes of several human and animal toxicoses throughout recorded history. Claviceps and Cordyceps were the sources of a treasure chest of medicines that have benefited millions of people. Clavicipitaleans have found applications in biocontrol of
insects, nematodes, and fungi. This chapter discusses the history of human relationship with various clavicipitalean fungi.

2. ERGOT AND HISTORICAL TOXICOSES
2.1. Biology of the Fungus

*Claviceps purpurea* has the highest profile of the species in the Clavicipitaceae, being the cause of countless human toxicosis. Sclerotia of *Claviceps*, formed in the rye (*Secale cereal*) inflorescence, were thought to resemble the spur on a rooster’s leg. The French word for spur, “ergot,” was adopted to refer to the sclerotia and the plant disease. The hosts of ergot include oats, rye, wheat, and numerous additional cool-season grasses. Ergot is biotrophic, with the fungus infecting ovaries and producing sclerotia that replace seeds. For this reason it is sometimes called a “seed replacement disease.” Ergot sclerotia are resistant, black, often cylindrical, bodies that function like seeds in that they overwinter and germinate in the spring to produce spores that set up new grass infections.

2.2. Ergot Alkaloids

Sclerotia contain a particular class of indole alkaloids referred to as “ergot alkaloids.” More than 50 different species of ergot alkaloids have been isolated from sclerotia of ergot. Ergot alkaloids have numerous effects on the physiology of animals that consume grain or products made from grain that contains sclerotia. Ergot alkaloids do not degrade when grain products are cooked. The biological functions of secondary metabolites such as ergot alkaloids are unknown, but one leading hypothesis suggests that they are defensive in nature, serving to protect sclerotia or the plant host from consumption (Clay, 1988). It is logical that they affect fungal physiology in some way, but this has not been well investigated. Effects of comparable secondary metabolites on ion channels may be a clue to their primary roles in the fungus (Garcia et al., 1996).

2.3. History of Ergotism

Ergot is likely to have been with humans since Euro-Mediterranean agriculture began (6000–2000 B.C.). However, the first documented epidemic of human toxicity that may be attributed to ergot occurred in what is now Germany in 857 A.D. Other epidemics followed in various places in Europe, regularly inflicting “plagues of fire” on human populations (Barger, 1931). An excerpt from one description of such an ergotism epidemic from 994 A.D. in south-central France is “and when a plague of invisible fire broke out, cutting off limbs from the body and consuming many in a single night, the sufferers thronged to churches and invoked the help of the saints” (Barger, 1931). Consumption of grain products containing ergot resulted in ergotism, either “convulsive” or “gangrenous.”
Gangrenous ergotism resulted from the vasoconstrictive properties of ergot alkaloids, which reduced flow of blood to the hands and feet. Affected people experienced a burning sensation as tissues of the hands and feet died and rotted due to insufficient blood flow. In addition, hallucinations and dementia were part of the symptoms of ergotism.

Ergot-induced hallucinations have been postulated to have resulted in the legends of werewolves, and visions of purgatory from the Middle Ages. Circumstantial evidence suggests that the witch hystrias in Europe and America were triggered by outbreaks of ergotism. The numerous witch trials of Europe occurred predominantly in areas where the populace relied on the highly ergot-susceptible rye as a staple. The symptoms of many of the “bewitched” individuals were consistent with ergot poisoning (Matossian and Caporael, 1976; Matossian, 1989). Disease epidemics in human populations through history, such as the Black Plague, have been suggested to have been facilitated by the weakening of humans by the long-term exposure to ergot alkaloids and other mycotoxins in bread that have the effect of weakening the immune system (Matossian, 1989). The demographic depression of human populations in Europe during the years 1350–1490 has also been hypothesized to be due to the reduced fertility of human females that consumed ergot-infected rye bread (Matossian, 1989). In 1765 Simon-André Tissot associated ergotism with the presence of the ergot fungus (Tissot, 1765). By the mid-nineteenth century ergotism in humans was less frequent, due to an understanding of the cause of ergotism and improved agricultural and milling practices.

3. ENDOPHYTES AND ANIMAL TOXICOSES

3.1. Biology of Epichloë/Neotyphodium Endophytes

Clavicipitaceous endophytes of grasses are almost exclusively species of Epichloë or anamorphs classified in Neotyphodium. These fungi grow in intercellular spaces of the above-ground organs of cool-season grasses. They are maternally transmitted from host generation to generation by infection of the embryo within the seeds of plants. Some of these endophytes have been shown to produce an epibiotic mycelium and conidia on the surfaces of leaf blades (Moy et al., 2000). It is unclear whether Epichloë/Neotyphodium endophytes may infect grasses using conidia produced on the surfaces of leaf blades.

3.2. Historical Toxicoses

Over the past two decades it has become clear that clavicipitatean endophytes have considerable economic impact through their effects on animal husbandry. This is particularly true for the grasses tall fescue (Festuca arundinacea) and perennial ryegrass (Lolium perenne), which are important pasture grasses and are
frequently infected by Neotyphodium coenophialum and *N. lolii*, respectively (Bacon et al., 1997). The endophytes of these grasses have only recently been recognized as the cause of animal toxicoses. Unexplained toxic syndromes in animals and people that consume particular grasses goes back to the beginnings of recorded history. Among grasses that have a long recorded history of association with toxicoses is “darnel” (*Lolium temulentum*), one of the annual ryegrasses. This grass is mentioned in the Old Testament or Hebrew Bible as undesirable (Kingsbury, 1964). In the gospel of Matthew (13:24–43), the gospel writer relates the parable of the “wheat and the tares” and emphasizes the practice of burning tares to remove them from the wheat. Darnel grew readily among wheat plants as a weed. For centuries it was known that contamination of flour with darnel seeds could result in human poisoning. The strong flavor and potential toxicity of darnel seeds reduced the quality of the flour. Darnel poisoning has been regional, with toxicoses reported exclusively from Europe, even though endophytes commonly grow in this species on all continents. The regional occurrence of toxicities is common with endophytes. Strain variation is believed responsible for this phenomenon. A similar phenomenon occurs in *Achnatherum robustum* infected with a *Neotyphodium* endophyte, for which toxic populations are recorded to occur only in New Mexico and Mexico, while endophyte infection is common throughout the range of the grass in the western United States (Vasey, 1887; Marsh and Clawson, 1929). Other writers from antiquity have also mentioned the darnel, including Dioscorides, Ovid, Shakespeare, and Virgil (Kingsbury, 1964). Symptoms of darnel poisoning in humans included apathy, giddiness, intoxication, ataxia, mydriasis, nausea, gastric pain, and diarrhea (Kingsbury, 1964). In the late 1800s darnel was discovered to be infected by *Neotyphodium endophytes* (Guerin, 1898). It has only been in the last 30 years that these endophytes have been demonstrated to be sources of a diverse array of secondary metabolites that cause toxic syndromes in grazing mammals (Lane et al., 2000).

### 3.3. Impact Worldwide

On a worldwide basis, several other clavicipitalean endophytes have affected humans by causing toxic syndromes in grazing animals. Among these are the sleepygrasses of North and Central America. One of these is *Achnatherum robustum*, locally abundant in certain places. One large population of sleepygrass is found in the mountains near Cloudcroft, New Mexico, at the Sleepygrass Campground. This campground, found along U.S. Highway 82, 1.5 mi east of Cloudcroft, acquired its name due to the repeated forced camps made by travelers. Riders passing through the mountains would permit their horses to graze on the abundant grasses in the area. After consumption of relatively small quantities of the grass, the horses would go to sleep for 2–3 days, then gradually
recover (Vasey, 1887; Havard, 1891; Bailey, 1903; Marsh and Clawson, 1929). Recent studies have identified the ergot alkaloid lysergic acid amide as the sleep-inducing agent in sleepygrass (Petroski et al., 1992).

In northwestern China and Mongolia the clavicipitalean endophyte-infected *Achnatherum inebrians*, or “drunken horse grass” as it is commonly known, has long been a nuisance (Hance, 1876). Upon consumption of the grass, horses develop a “staggers” that make it difficult for them to stand. Severely affected horses die within 24 h of consumption of the grass. Bruehl et al. (1994) confirmed the presence of a clavicipitalean endophyte in the grass. Miles et al. (1996) identified lysergic acid amide and ergonovine as two toxic endophyte-produced components of the grass. Drunken horse grass has become a recent concern due to its increasing range expansion. The distribution of the grass was once limited to semiarid lands at around 1500-m elevation. However, in the 1980s it was found to be spreading into lower- and higher-altitude grazing lands. Toxicosis due to the plant is rare because animals local to the region where the grass occurs avoid it, apparently because they can taste or otherwise detect the toxins. Under the intense grazing pressure that is occurring currently on Chinese and Mongolian grazing lands, the avoidance of drunken horse grass may provide the selective advantage that is allowing the plant to expand its range. Populations of the Asian *Achnatherum sibericum* have also been reported to cause toxicities in animals in certain locations (Rau, 1975). *Neotyphodium* endophytes have been demonstrated to occur in this species (White et al., 2001) but studies on toxins have not been made. In South Africa another grass, “dronk grass” (*Melica decumbens*), contains a clavicipitalean endophyte that produces symptoms similar to those of drunken horse grass (Shaw, 1873). Miles et al. (1995a, b, c) reported that this grass contains indole-diterpenoid tremorgens. Serological tests were used to confirm that the endophyte was clavicipitalean. In Australia a clavicipitalean endophyte was found to cause a staggers toxicosis in animals that consumed the native grass *Echinopogon ovatus* (Miles et al., 1998).

The endophyte *Neotyphodium tembladerae* is widespread in several grasses of South America (Cabral et al., 1999). Rivas and Zanolli (1909) linked presence of the endophyte in the andean grass *Festuca hieronymi* with a “staggers” condition called “tembladera” in Argentina. *Neotyphodium tembladerae* produces the ergot alkaloids ergovaline and peramine (White et al., 2001), but lolitremes have also been detected (Miles et al., 1995a, b, c). One common name for *Poa huecu* is “huecu,” meaning “intoxicator” in the indigenous Araucanian language that was spoken by tribes that lived in the region (Parodi, 1950). *Poa huecu* is frequently lethal to animals. Pomilio et al. (1989) identified glycoproteins as toxic components of *N. tembladerae*-infected grasses. It has been reported that scavengers that consume the bodies of intoxicated herbivores will themselves be poisoned (Parodi, 1950).
In Argentina, the endophyte *Neotyphodium tembladerae* has been employed as a weapon of defense against armies or soldiers on horseback. In what has been termed the “strategy of huecú,” Indians, soldiers, or bandits fleeing pursuers would purposefully enter zones dominated by *P. huecu*. Those employing the “strategy of huecú” had the knowledge to prevent their horses from consuming the toxic grasses. However, their pursuers were frequently unfamiliar with huecú grass and would permit feeding by their animals. The rapid intoxication and death of the pursuers’ animals usually permitted escape (Nicora, 1978).

4. ERGOT, ENLIGHTENMENT, AND REVELATION

4.1. The Arguments for Use of Ergot

Huston Smith (2000) argued that many religions were built on religious experiences facilitated through use of various consciousness-altering natural products. There have been numerous speculations that ergot was among the natural intoxicants that humans have used to try to “bridge the gap” between humans and God. Most of these speculations are built on circumstantial evidence and thus open to doubt. The absence of explicit written records identifying ergot sclerotia as an active ingredient in any ceremony to commune with God, nature, or gain spiritual or intellectual enlightenment keeps a shadow of doubt on all hypotheses regarding such uses for sclerotia. Nevertheless, historians and mycologists have persisted in proposing hypotheses regarding ancient spiritual and religious applications of ergot. Such uses of ergot by people are reasonable because: (1) people likely routinely collected ergot sclerotia from grain as they cleaned it and massive quantities would be gathered; (2) ergot contained psychotropic alkaloids; and (3) people tend to experiment and would likely have sought applications for the sclerotia and eventually discovered their properties. The absence of explicit records of use of ergot is not unexpected when one considers that “magic” or “sacred” potions or preparations were the domain only of specialized people in a society. The people who employed such “magic” were not disposed to record their secrets for the world to learn, and why should they, since it was the magical or divine experience that was reality. The ingredients employed were largely irrelevant and served as nothing but aids for the magical or divine experience.

4.2. The Eleusinian Mysteries

Wasson et al. (1978) argued that the Greek Eleusinian mysteries involved the use of an aqueous concoction of ergot sclerotia that contained water-soluble alkaloids, perhaps including lysergic acid diethylamide (LSD). These mystical ceremonies were held in ancient Greece at Eleusis, near Athens. The mysteries
involved visitation of the Greek goddess Persephone. In preparation to encounter the Eleusinian mysteries, initiates would fast and make sacrifices. The fast was broken by drinking “kykeon,” a purple-colored potion containing meal (presumably ground sclerotia), water, and mint flavorings. The experience was not pleasant, involving sweating, trembling, vertigo, hallucinations, and fear (Bennett and Bentley, 1999). The Greek philosophers and writers Euripides, Homer, Plato, Socrates, and Sophocles wrote of the Eleusinian mysteries. It is possible that philosophical and literary advancements made by these intellectuals were influenced by psychotropic experiences induced by ergot alkaloids.

4.3. Was the Manna of the Israelites Ergot?

Daniel Merkur (2000) has espoused the hypothesis that the “manna” of the Israelites was a mixture that contained ergot. According to this hypothesis, manna aided the Israelites to experience the supernatural presence of God. The use of such psychotropic manna extended into religious experiences of the early Christian era, with events such as the descent of the Holy Spirit to the disciples, speaking in tongues, etc., being alkaloid-facilitated experiences.

The mystery of manna and the Eleusinian mysteries remain mysteries because of scant literary records. Uncertainty is magnified because no experimental studies have been conducted to evaluate whether primitive techniques for extracting or modifying bio-active components from ergot could have yielded preparations with the purported properties of “manna” or “kykeon” without causing destructive ergotism.

4.4. The Great Awakening

Mary Kilbourne Matossian (1989) argues persuasively that the “Great Awakening,” a religious revival that peaked in 1749, in the colonies of New England was stimulated by outbreaks of ergotism from contaminated rye. During the Great Awakening many people in the colonies experienced visions (hallucinations) and other symptoms of ergotism. Many people in the colonies gave a religious significance to the symptoms, interpreting them as divine. Matossian’s arguments are based on written accounts by physicians and others who experienced the events. A description of events is provided by the Reverend Charles Chauncy (1743), an opponent of the revival movement:

When they come out of their trances they commonly tell a senseless story of heaven and hell, and whom and what they saw there. In some towns, several persons, both men and women, that formerly were sober, and to all appearance truly pious, are raving distracted, so that they are confined and chained. Many fall into epilepsies, as they walk in the streets, or in their houses. Many people experienced a sudden and terrible fear of divine
wrath, or the miseries of hell, occasioning a sensation of cold, in most a very extraordinary warmth all over the body; causing people to cry as if distracted; to shed tears in great plenty; throwing many into convulsions, and a few for some time into despair.

The Reverend’s candid description of the “religious experience” that he observed is a pretty good account of convulsive ergotism. Many other accounts of “religious experiences” from the period of the Great Awakening also are consistent with a mass ergotism epidemic at the time.

Based on the studies of Matossian (1989), it is tempting to speculate that many of the “religious experiences” through recorded history coming from the European and North American Christian churches, where people depended on rye as a predominant food source, were in fact ergot toxicoses given religious interpretation.

4.5. The Ergot Enlightenment

In the 1960s, the “high priest” of the psychedelic movement, Timothy Leary, and many others advocated the ergot alkaloid derivative LSD as a means to self-enlightenment. The philosopher and writer Aldous Huxley, along with many other philosophers, artists, and writers of the time, experimented with LSD and comparable compounds to enhance consciousness. Their view was that waking consciousness is only one form and that enlightenment can be found in experiencing other forms of consciousness. This view was articulated by William James a century ago as follows:

Our normal waking consciousness is but one special type of consciousness, whilst all about it, parted from it by the filmiest of screens, there lie potential forms of consciousness entirely different. We may go through life without suspecting their existence; but apply the requisite stimulus, and at a touch they are there in all their completeness. No account of the universe in its totality can be final which leaves these other forms of consciousness quite disregarded (James, 1902).

The quest to explore other levels of consciousness was greatly facilitated by the discovery of LSD.

5. CLAVICIPITALEANS IN MEDICINE

5.1. History of the Medicinal Use of Ergot

Ergot figures among the earliest known medicinal natural products. Ergot was first mentioned in herbal medicine in the late sixteenth century (Barger, 1931). European midwives used sclerotia of Claviceps purpurea to hasten childbirth. Consumption of a few sclerotia frequently reduced the time of delivery by hours.
It was documented to be in use in the United States in the early nineteenth century. Dr. John Stearns recommended ergot for its oxytoxic properties, reporting that since he began using ergot, “I have seldom found a case that detained me more than three hours” (Barger, 1931). The 1836 *Dispensatory of the United States* recommended 15–20 sclerotia to induce uterine contractions (Riddle, 1997). The 1839 French *Codex* required that ergot sclerotia be maintained in pharmacies (Bennett and Bentley, 1999). Ergot was also capable of inducing abortion of the fetus in pregnant women at all stages of the pregnancy, and it is probable that midwives used ergot’s abortifacient properties (Riddle, 1997). Because of strain variation among collections of sclerotia and the inexact means by which women were dosed with sclerotia, the dose of alkaloids actually delivered to women was highly variable. The result was that women and infants sometimes died in the process. Bennett and Bentley (1999) suggested that the use of ergot and other dangerous natural products by midwives contributed to the midwives being stigmatized as witches.

### 5.2. Medicinal Psychedelics

It was not until the early twentieth century that the active ingredients in ergot sclerotia were purified and identified. Arthur Stoll (1918) patented the isolation of ergotamine tartrate from sclerotia. The Swiss company Sandoz introduced ergotamine tartrate to the market in 1921. This was followed by purification and characterization of other ergot alkaloids, including ergocristine and ergocristine (Stoll and Burckhard, 1937). Purification and characterization of ergot alkaloids was continued by many investigators, including Albert Hofmann at Sandoz, who was in part responsible for its development as a recreational drug (Hofmann, 1964, 1980). Albert Hofmann discovered LSD$_{25}$, number 25 in a series of semisynthetic derivatives of lysergic acid diethylamide. As the story is told, one day in April of 1943, Hofmann began to feel dizzy and lightheaded. He managed to get home on his bicycle. In reflecting on what happened, he suspected that it must have to do with his current work on LSD derivatives, which had been absorbed through his skin. He initiated a series of self-experiments with LSD (Hudler, 1998). Soon the whole world was participating in Albert Hofmann’s experiments and the psychedelic era was initiated.

### 5.3. Other Ergot Alkaloid Applications

Many of the ergot alkaloids or their derivatives have been employed medicinally. Ergotamine tartrate is prescribed for migraine headaches. Migraines are in part due to the vasodilation of blood vessels in the brain, causing pressure on tissues of the brain. The vasoconstrictive properties of ergonovine helps to relieve the pressure by constricting the blood vessels. The derivative methysergide is used in early stages of migraines. Bromocriptine, a derivative of ergocryptine, suppresses
several hormones and is used to treat acromegaly and hyperprolactinemia, both due to excess secretion of hormones. Bromocriptine is also used to treat Parkinson’s disease, due to its stimulation of dopamine receptors. Because of dopamine enhancement and prolactin suppression, bromocriptine enhances sexual desire and enjoyment. As a result it has acquired the status of a “love drug.” It has been reported that sexual orgasms are more intense with bromocriptine treatment, being described as increasing in a stepwise manner with a crescendo of minor almost-orgasms, and culminating in a final orgasm punctuated by the release of histamine (Anonymous, 2000). Hydergine (Novartis, formerly Sandoz, Switzerland) is another ergot alkaloid product that is a mixture of three semisynthetic alkaloid derivatives. This drug is used as a treatment for senility and dementia due to aging and decreased blood flow to the brain. It has also become a popular “smart pill.” Life extensionists have popularized the drug as a means of extending brain health and function beyond the average number of years (Pearson and Shaw, 1982). Hydergine increases brain function by increasing blood flow and nerve growth in the brain. This has the effect of improving memory and brain function. Several studies have also suggested that hydergine protects the brain from damage under oxygen deprivation conditions, such as during a stroke. Hydergine is in high demand as a brain rejuvenator and protector and is readily obtained from vendors on the Internet without a prescription. Many additional ergot alkaloids are known and have been produced industrially for various applications (Cvak, 1999).

5.4. A Breakthrough for Transplant Surgery

Several other clavicipitalean fungi have also been employed for their medicinal properties or constituents. In 1976 scientists at Sandoz were responsible for isolating and purifying cyclosporin A (Sandimmune, Novartis) from the soil fungus *Tolypocladium inflatum* (the conidial state of *Cordyceps subsessilis*). Cyclosporin A is a cyclic peptide that has been found to be a potent immune suppressant. This compound is employed to suppress the human immune systems of organ transplant patients so that organ rejection does not occur. Cyclosporin made possible the recent advances in the science of organ transplantation. Cyclosporin is also somewhat effective in the treatment of diabetes and certain auto-immune skin disorders.

5.5. The Caterpillar Mushroom

*Cordyceps sinensis* is the source of cordycepin, an antibiotic and one of the active compounds in the fungus. In China *C. sinensis* is commonly called the “caterpillar mushroom” and has been used as a medicinal since antiquity. A water extract of *C. sinensis* is believed to build immune function and improve athletic endurance and performance (Hobbs, 1995).
5.6. Endophyte Sleeping Aid

Clavicipitalean endophytes (*Neotyphodium* spp.) in sleepygrasses (*Achnatherum robustum*) have been used medicinally as sleeping aids in Native American cultures. Emboden (1976) reported that North American and Central American Indians used sleepygrasses (known by the Nāhuatl-derived name “popoton sacaton,” meaning sleepygrass) as hypnotics and as sleep-inducing agents. Emboden (1976) seems to suggest, perhaps because of the Aztec name, that use of sleepygrasses goes back at least to the time (~1100–~1500 A.D.) of the Aztecs. However, the use of this “nutraceutical” sleeping aid by natives in Guatemala (Emboden, 1976) suggests that it may have been acquired by the earlier Mayan civilization and spread through the Mayan Empire down into Central America perhaps as early as ~2500 B.C. It is certainly possible that the Mayans actively cultivated and spread sleepygrass throughout their empire for its medicinal properties. If this hypothesis is correct, the endophyte-infected sleepygrass could be among the earliest known of the medicinal plants and may be the first “sleeping aid.”

5.7. Use of *Balansia cyperi* by Amazonian Jivaro

The Amazonian Jivaro tribe, well known for their witchcraft and “head shrinking,” use clavicipitalean fungi in their medicine (i.e., magic). Lewis and Elvin-Lewis (1990) reported that the Amazonian Jivaro women employ the clavicipitalean *Balansia cyperi* in a comparable way to how midwives and Western doctors used ergot. They prepare a water infusion of crushed mycelial stromata of the fungus that covers inflorescences of locally abundant sedges. The infusion is then consumed by females who are giving birth, to facilitate the birthing process.

6. CLAVICIPITALEANS AS BIOCONTROL AGENTS

6.1. Epichlöë/Neotyphodium Endophytes in Biocontrol

Clavicipitaleans are important as biological control agents. The clavicipitalean endophytes of grasses are notable in that over the last two decades they have been shown to have applications in improvement of hardiness of commercial grass cultivars (Funk and White, 1997). In tall fescue, *Neotyphodium coenophialum* increases insect and nematode resistance and makes the grass more tolerant to drought conditions. The increased hardiness of the grass means that chemical inputs required for maintenance of stands of the grass are reduced. Further, enhanced drought tolerance allows cultivation of tall fescue cultivars with the endophyte in drier areas. Perennial ryegrass is infected with an endophyte, *Neotyphodium lolii*, that provides significantly enhanced resistance to insects. In New Zealand, endophyte-free ryegrass cannot be cultivated, due to devastation
by the Argentine stem weevil. However, *N. lolii* provides an almost absolute resistance to the Argentine stem weevil. Ranchers are forced to feed cattle endophyte-infected ryegrass and manage toxicosis, called “ryegrass staggers.” Because of this “catch-22” situation, considerable effort has been expended in New Zealand to identify endophytes that do not cause ryegrass staggers but retain insect-resistance properties. This is possible because, at least in some cases, the animal toxins and insect deterrent properties stem from different compounds. The mechanism of increased drought tolerance is still not understood (West et al., 1990). Endophytes in fine fescues have been shown to make grasses resistant to fungal diseases of leaves, perhaps due to a competitive exclusion phenomenon (Moy et al., 2000). Endophytes in fine fescues and many other grasses have been found to produce an epiphyllous stage on the surfaces of the plant. In many grasses these epiphyllous stages form a branching network of mycelium, termed “defensive net,” that covers leaf blades and may play a role in exclusion of colonization of the leaves by pathogens. Yue et al. (2000) demonstrated that many of the secondary metabolites produced by the fine fescue endophyte *Epichloë festucae* were inhibitory to other fungi. Thus it is possible that secondary metabolites produced by endophytes may serve as antifungal compounds in the active defense of leaves from pathogenic fungi. Regardless of the precise mechanisms by which endophytes impart benefits to their grass hosts, they have stimulated interest and excitement as examples of “defensive mutualisms.” Clay (1988) and many other researchers (Bultman et al., 1997; Reddy et al., 1996; Schardl and Phillips, 1997; Tibbets and Faeth, 1999) have developed a base of evidence that supports a defensive mutualism role for clavicipitalean endophytes.

### 6.2. Insect Biocontrol

Insect-infecting clavicipitaleans figure prominently as biocontrol agents. Among these are *Metarhizium anisopliae* and *Beauveria bassiana*, both of which are asexual expressions of species of genus *Cordyceps*. These fungi have found applications in the large market for effective biocontrol agents among home gardeners and organic farmers. Commercial preparations of these fungi typically consist of dried conidia that may be applied to plant surfaces. Interestingly, recent studies on *B. bassiana* have shown that, after application to plants, the fungi infect plants to form an endophytic stage (Bing and Lewis, 1992). After consumption by insects, these fungi parasitize and kill the host.

*Beauveria bassiana* has the added distinction of being the first demonstrated “pathogen.” From antiquity into the nineteenth century it was generally accepted that disease was caused by supernatural or unseen agents and that diseased or rotting bodies of animals and plants spontaneously generated microbes or other forms of life. That the microbes themselves were the cause of
disease, known as the “germ theory of disease,” was first demonstrated by Agostino Bassi, an Italian lawyer turned scientist. Bassi investigated the disease of silkworms called “mal de segno” or “muscardine disease.” After 25 years of research on this disease he demonstrated that it was caused by a contagious fungus. The scientific name *B. bassiana* was given in his honor. Bassi (1835) further proposed that many diseases of animals, plants, and humans were caused by parasites. Bassi preceded Pasteur, Koch, and others in espousing the germ theory of disease.

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2

A Taxonomic Review of the Clavicipitaceous Anamorphs Parasitizing Nematodes and Other Microinvertebrates

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1. INTRODUCTION

The association between nematodes and fungi that colonize them has been the subject of extensive mycological studies. The voluminous and meticulous work by Charles Drechsler (1892–1986; see references) has laid the foundation for our knowledge of nematophagous fungi. Numerous publications by Barron (since about 1969, particularly 1977, 1981, 1982, and other papers cited) subsequently augmented our knowledge about the diversity and distribution of these fungi. A detailed understanding of their classification in a teleomorph-based fungal system is emerging only in recent years.

The nematophagous fungi in the broadest sense (for recent reviews see, e.g., Barron, 1977, 1981, 1982; Gray, 1988; Kerry and Jaffé, 1997) comprise zoosporic, zygomycetous (Zoopagales), ascomycetous (anamorphic Orbiliaceae and Clavicipitaceae), and basidiomycetous (Pleurotaceae) representatives. In a
first instance, they are subdivided into nematode-capturing fungi, endoparasites of the free-living animals, and cyst and egg parasites. While nematode-capturing capacities are found mainly in some Zoopagales, the ascomycete family Orbiliaceae and basidiomycetes of *Hohenbuehelia*, the majority of nematode endoparasites and egg parasites belong to the Clavicipitaceae and associated anamorph taxa. Not only nematode-capturing but also endoparasitic parasites attract nematodes (Jansson and Nordbring-Hertz, 1988). The endoparasites colonize the animal from small propagules, produce internal mycelium, and finally penetrate the corpse to sporulate on its surface. Endoparasites generally employ two modes to penetrate the host animal, oral ingestion of conidia which germinate in the intestines (mostly the esophagus or mastax), or direct penetration of the cuticle from conidia which adhere firmly to the surface. This adhesion occurs only in certain combinations of host and parasite taxa as reviewed below for *Drechmeria*, *Haptocillium*, and *Hirsutella*. From the attached conidium a narrow penetration tube grows through the nematode cuticle, apparently with some mechanical pressure (Dackman et al., 1992; Dijksterhuis et al., 1994). Egg parasites will be treated further below. Besides nematodes, mainly terrestrial bdelloid rotifers (genus *Adineta*), some ciliated Protozoa, and rarely Tardigrada have been found to be colonized by similar fungi.

The nematode body is covered by a thick proteinaceous cuticle, the eggs by lipid and chitin layers, and an outermost protein layer characterized as vitellin (Jatala, 1986; Bonants et al., 1995), and the cysts of certain genera by protein and mucopolysaccharide. These recalcitrant layers provide barriers to penetration that can be crossed only by enzymatically qualified fungi (Morgan-Jones et al., 1983; Stirling, 1991). Strong chitinolytic activities are observed in many of the fungi involved, which share this capacity with most entomogenous and many fungicolous fungi. It has been hypothesized, in a phylogenetic analysis of *Cordyceps* species, that a jump from entomogenous to fungicolous lifestyle is possible within a certain common biotope (Nikoh and Fukatsu, 2000). Therefore it is conceivable that certain endoparasites could also have jumped from insects to nematodes or other animals. Such a jump has been suggested to have occurred within a species of *Lecanicillium* (Hall, 1980) or between closely related taxa. Some of the species to be mentioned have also been isolated as apparently saprotrophic soil fungi.

Nematodes are subdivided into saprotrophic or bacterial feeders, plant-pathogenic, and predatory groups. Parasites affecting the second group have most frequently been studied in view of their possible role in biological control. However, the plant-feeding nematodes have a specialized narrow mouth apparatus with a stylet, which prevents ingestion of fungal propagules. These animals can only be attacked in their free-living stage by fungi that produce adhesive conidia. Bacterial feeder nematodes are much more prone to parasitism by both nematode-capturing and endoparasitic fungi. They can ingest conidia of
their parasites, such as *Harposporium* species, through their wider mouth openings. For the adhesion of conidia of *Drechmeria* (or to a lesser extent of *Haptocillium*) to specific parts of the body, chemical factors are responsible, which are still not sufficiently elucidated (see below under *Drechmeria*). The difference between bacteriophagous and phytophagous nematodes is of great ecological significance in relation to endoparasitic and other nematophagous fungi. The fact that we see relatively little host specificity is inconsistent with the “host relatedness hypothesis” proposed for endoparasites (Nikoh and Fukatsu, 2000). Particularly in *Harposporium* and *Rotiferophthora*, a broad range of taxa occurs on a rather limited array of prey animals.

In contrast with the numerous free-living nematodes, some plant-pathogenic nematodes spend most of their life inside plant roots or on their surface in cysts and/or root knots. These resting structures persist in the soil and act as a selective substratum for fungal colonization by egg parasites. Certain clavicipitaceous anamorphs, now comprised in the genus *Pochonia* (Zare et al., 2001), are specialized to parasitize such resting structures (for a review see Morgan-Jones and Rodríguez-Kában, 1988). In addition, the free-living phase of the same nematodes can be attacked by a different array of parasites, mainly of *Haptocillium* and *Hirsutella*.

Species of *Pochonia, Haptocillium*, and *Hirsutella* are therefore among the most promising biocontrol agents against plant-pathogenic nematodes (Dackman et al., 1992; Kerry, 1987, 1989, 1990, and later work; Tribe, 1979, 1980; Stirling, 1988, 1991). Such endoparasitic fungi are often considered more amenable to practical applications than nematode-capturing fungi (Persmark et al., 1996).

Biological control can manifest itself in three situations: (1) natural control, which includes decline phenomena, is mainly depending on soil characteristics; (2) addition of antagonists together with additional nutrient sources, which often requires application of prohibitively high quantities of inoculum; or (3) the stimulation of the resident population of antagonists, e.g., by organic amendments or various cropping systems (Dackman et al., 1992; Stirling, 1989, 1991). Practical approaches to biological control therefore include the exploitation of naturally suppressive soils, soil amendments to encourage the activity of indigenous nematode parasites, application of selected strains of bacteria or fungi, and the application of fungal toxins and enzymes (Jansson et al., 1997; Kerry, 1990; Stirling, 1989, 1991). Seed treatment is considered less feasible for these fungi because of poor reproduction in the rhizosphere (Kerry and Jaffee, 1997). A fungus, *Pochonia chlamydosporia*, and a bacterium, *Pasteuria penetrans*, are regarded as promising control agents in small-scale tropical agriculture with low input of chemicals (Davies et al., 1991).
2. METHODS OF STUDY

Methods of handling nematophagous fungi have been detailed by Duddington (1955), Barron (1969, 1977, 1981, 1982), Gray (1984), and Bailey and Gray (1989). The sprinkled soil plate method introduced by Drechsler (1933) and frequently modified (e.g., Barron, 1977) retrieves all kinds of nematophagous fungi. Particles of soil or various organic substrata are spread onto water agar or a dilute cornmeal agar, and nematodes (particularly *Panagrellus redivivus*, *Turbatrix aceti*, or others) are added as baits. The plates are checked at weekly intervals for fungi growing on or out from dead nematodes. To retrieve endoparasitic species preferentially, the Baerman funnel technique, adopted for this purpose by Giuma and Cooke (1972), is significantly more effective (Barron, 1977, 1978, 1982; Gray, 1984). However, both methods are usually employed together to reveal the maximum of taxa present in a soil. A differential centrifugation technique was proposed by Barron (1969): the supernatant of a soil suspension obtained after gentle centrifugation is supposed to contain most conidia; when spun down at high speed, the pellet obtained from the first supernatant can be plated on agar with nematodes. Subsequent comparative studies by Barron (1978) and Gray (1984) showed that this technique had little additional advantage over the Baerman funnel. Dackman et al. (1987) combined a dilution plate method (otherwise similar to the soil sprinkling method) with most probable number analysis to quantify populations of nematode parasites. Persmark et al. (1996) almost exclusively obtained zoosporic parasites with this method and therefore discontinued its use. Banck et al. (1990) compared methods for retrieving plant-parasitic nematodes and their parasites. They retrieved species of *Harposporium* and *Hirsutella* by Seinhorst’s elutriation and Cobb’s sieving, decanting, and centrifugation method with either silica or sugar solutions. To detect ovicidal fungi in soil, Fassatiová and Lysek (1982) buried eggs of *Ascaris lumbricoides* in soil.

To recover parasites of bdelloid rotifers from samples of soil or organic debris, Barron (1985) used cultured rotifers of the genus *Adineta* (originally recovered from sprinkled-soil plates as used for nematophagous fungi) as bait. The animals are maintained in Petri dishes in a thin film (2 mm) of water or physiological saline layered over sucrose-free Czapek agar. Cultures of rotifers are maintained by periodic transfer (1–2 weeks) to fresh Petri dishes. From 10 to 25 g of soil or organic debris are mixed with an equal volume of sterile water in a plastic bag and squeezed and agitation vigorously for several minutes. Then 1–2 mL of the slurry is transferred to a rotifer culture in a Petri dish and swirled gently. The dishes are incubated at 18–22°C and examined at weekly intervals for 3–6 weeks. Infected rotifers can be spotted under a dissecting microscope by a cluster of conidiophores arising from the floating body of a dead rotifer. These rotifers are
then transferred with a flattened needle to a fresh rotifer culture; new crops of infected rotifers will appear in 3–7 days and can be used for preparing pure cultures.

For methods developed for specific taxa, see the texts under each genus.

Mass production of inoculum for nematode control has not yet been upscaled industrially. To assure optimal longevity and infectivity of the conidia, the fungi are generally grown in solid-state surface cultures; see under *Pochonia chlamydosporia*. For the production of *Hirsutella rhossiliensis* inoculum, stirred cultures in 5-L containers were used (Patel et al., 2001).

### 3. ECOLOGY

The ecology of nematophagous fungi has been reviewed extensively (Duddington, 1951; Barron, 1977, 1981; Gray, 1983a, 1984). Soil and various organic substrata, particularly dung (Juniper, 1967; Glockling and Yamada, 1997), are suitable sources for nematophagous fungi. Extending this range, Gray (1983b) found nematode endoparasites in deciduous and conifer litter, old dung, moss cushions, and decaying vegetation. Addition of farmyard manure to agricultural soil increased the population of endoparasites (Dackman et al., 1987).

Densities of *Harposporium anguillulae* in a Swedish agricultural soil reached maxima in March and June. The highest densities of nematophagous fungi in general were found in the upper 40 cm, with *Harposporium anguillulae* going down to 30–40 cm and *Hirsutella rhossiliensis* strongly declining after 20 cm. During fallow periods the population of nematode parasites declined (Persmark et al., 1996). Certain nematophagous fungi were stimulated under plants with a strong rhizosphere effect, particularly peas; among these, *Harposporium anguillulae* was found on extracted nematodes in 4 of 5 soils examined, and *Haptocillium balanoides* in one rather acidic soil, both grown with barley (Persmark and Jansson, 1997).

Nematodes are attracted to colonies of *Drechmeria coniospora*, *Haptocillium balanoides*, and other endoparasites (Barron, 1982; Jansson, 1982a, 1982b; Jansson and Nordbring-Hertz, 1979, 1980). As a mechanism of nematicidal action, antibiotic (antifungal) activities have been demonstrated for *Drechmeria coniospora*, *Harposporium anguillulae* (Barron, 1977), *Lecanicillium* (Hänssler, 1990), *Paecilomyces lilacinus* (Jatala, 1986), and *Pochonia* (Segers et al., 1999).

Even the large eggs of *Ascaris* can be parasitized when exposed in soil. Fassatiová and Lysek (1982) obtained *Pochonia chlamydosporia*, *P. bulbillosa*, *Paeicilomyces marquandii*, *P. lilacinus*, and *P. carneus* from such eggs buried in soils in the Czech Republic, Pakistan, Afghanistan, and Cuba. *Pochonia* spp. and *P. lilacinus* rapidly infected and killed the eggs.
4. DISTRIBUTION

Most species of nematode endoparasites were originally described in the United States and Canada. Reports from other countries, however, indicate an almost cosmopolitan distribution, but a few species are either tropical or temperate. Species lists have been compiled from Ireland (Gray, 1983b), New Zealand (Hay, 1995), and El Salvador (Bucaro, 1983); and from vegetation and soils in the maritime Antarctic (Gray, 1982; Gray et al., 1982; Gray and Lewis Smith, 1984). 

*Pochonia chlamydospora* is one of the most cosmopolitan species, but its *Cordyceps* teleomorph is so far known only from slug eggs in the tropics (Zare et al., 2001). *Drechmeria coniospora, Haptocillium balanoides, Harposporium anguillulae*, and *Hirsutella rhossiliensus* were also encountered infrequently in Central America (Persmark et al., 1995).

5. MORPHOLOGY

In axenic culture, colonies are slow- to medium-fast growing (reaching 5–40 mm in diameter in 10 days at about 20°C on common laboratory media, depending on the taxon), white to yellowish, with some cottony aerial mycelium, often consisting largely of fertile hyphae or conidiophores. In most taxa conidiophores are at least partly verticillate, either erect or soon procumbent or prostrate, so that indefinite numbers of phialides can arise from arched hyphae of the aerial mycelium. Conidiogenesis is mostly phialidic; sometimes only solitary conidia are formed on a conidiogenous cell, i.e., blastic conidiogenesis. Phialides are more or less swollen in the lower part or aculeate, with a single (monophialide) or several conidiogenous tips (polyphialides or polyblastic conidiogenous cells). If the conidiogenous cells are phialidic, they produce usually several conidia in slimy heads, sometimes in fascicles that aggregate at the tip in a position perpendicular to the phialide (characteristic of *Lecanicillium*). Conidia are mostly one-celled, of various shapes and sizes. Some taxa produce complex resting and propagative structures called dictyochlamydospores, i.e., hyaline, thick-walled, pluricellular bulbil-like structures supported by a short stalk. The development of the characteristic dictyochlamydospores was studied by Campbell and Griffiths (1975). These dictyochlamydospores can also be absent in some strains of species supposed to produce them, but then some irregularly swollen, thick-walled intercalary cells are usually present in the vegetative hyphae.

6. TAXONOMY

The taxonomy of clavicipitaceous nematode parasites has been considerably modified in recent years, leading to the distinction of several phylogenetically distinct genera. *Drechmeria* was segregated from *Meria* (Gams and Jansson, 1985); its unrelatedness with *Meria* Vuill. (Rhytismatales) and its affinity with
the Clavicipitaceae has been proven (Gernandt and Stone, 1999). The type species of *Tolypocladium* was connected with the teleomorph *Cordyceps subsessilis* Petch (Hodge et al., 1996), but the nematophagous species of *Tolypocladium* are not yet critically classified. Some species demonstrate a continuum between the genera *Harposporium* and *Hirsutella*, producing two kinds of conidia with the associated kinds of conidiogenesis (Hodge et al., 1997; Glockling 1998b). The species of the former *Verticillium* sect. *Prostrata* W. Gams (1971) comprised several groups of nematophagous taxa as reviewed by Gams (1988), but molecular analyses (Zare et al., 2000; Sung et al., 2001) have shown that members of this section are heterogeneous and must be distributed among several genera (Gams and Zare, 2001), a classification that is adopted here and elaborated below in reference to microinvertebrate-parasitizing taxa. Barron (1991a) had already singled out the very slowly growing rotifer parasites in a separate genus, *Rotiferophthora* Barron. The most characteristic nematophagous verticillium-like genera are *Haptocillium* W. Gams & Zare, comprising species with adhesive conidia that stick to free-living nematodes, and *Pochonia* Bat. & O. M. Fonseca, formerly often called *Diheterospora* Kamyschko ex Barron & Onions, species which are particularly capable of penetrating nematode cysts and eggs. The production of dictyochlamydospores was mostly used to characterize *Diheterospora*, but this is an unreliable criterion for recognizing species of this genus, because they are absent or scanty in some species, while similar structures also occur in species of *Rotiferophthora* and *Haptocillium*. The present separation of several verticillium-like genera has the advantage of reflecting correlated ecological, morphological, and phylogenetic traits.

Methods for identification can be summarized as follows. Colonies can be grown on various media that are not too rich in nutrients, such as cornmeal agar (Difco), a dilute oatmeal agar (OA), malt extract agar (not more than 2% sugar), synthetic nutrient-poor agar (SNA), potato-carrot agar, or even water agar (Gams et al., 1998a). Transfer by streak inoculation is recommended to induce good, homogeneous sporulation from colonies developing from a conidial inoculum. Direct observation of the undisturbed colony in the open Petri dish under the compound microscope allows the observation of the branching system and structures of the conidiophores and the arrangement of the conidia in situ. Microscopic mounts are made in lactic acid (sometimes with cotton blue or a similar stain) and recorded in camera-lucida drawings or photographs.

7. KEY TO THE GENERA THAT INCLUDE PARASITES OF NEMATODES AND OTHER MICROSCOPIC ANIMALS

In this general survey, we illustrate representative species of each genus mainly with drawings taken from original publications. In the special part we add some drawings of our own.
1. Conidiogenous cells with almost globose venter and sharply delimited slender neck........................................................................................................................................2

1'. Conidiogenous cells aculeate or with moderately inflated venter or of reduced shape..................................................................................................................................................3

2. Conidia bearing curved phialoconidia on one or several necks (cylindrical in some Harposporium species); generally parasitizing nematodes or bdelloid rotifers ......................5. Harposporium (Fig. 1)

FIGURE 1 Harposporium, conidiophores arising from infected nematodes and conidia: (a) H. anguillulae. (From Zopf, 1888.) (b) H. helicoides. (From Drechsler, 1941.)
2'. Conidia bearing globose to cylindrical conidia, usually from single necks; soilborne or entomogenous, rarely associated with rotifers .................................................. 14. *Tolypocladium*

3. Intercalary conidiogenous cells (mostly phialides) with short conidiiferous necks commonly produced ................................................ 4

3'. Intercalary conidiogenous cells absent (or rarely formed in *Haptospora*) .................................................. 6

4. Intercalary phialides mostly produced singly, frequently in verticillate end-branches of the conidiophore; a conspicuous oil globule present in each conidium; parasites of rotifers; dictyochlamydomspores present ......................... 12. *Rotiferophthora* (Fig. 2)

4'. Several intercalary conidiogenous cells produced below a terminal one; dictyochlamydomspores absent .................................................. 5

5. Conidiogenesis phialidic with single openings; conidia obclavate with adhesive tip; parasitizing nematodes or ciliated protozoa ...........

................................................................. 2. *Drechmeria* (Fig. 3)

5'. Conidiogenesis polyblastic; conidia globose, with a conspicuous basal slime pad; parasitizing rotifers 11. *Pseudomeria mucosa* (Fig. 4)

6. Conidiogenous cells with more or less swollen venter; discrete dictyochlamydomspores absent .................................................. 7

6'. Conidiogenous cells hardly swollen, aculeate, often in whorls; dictyochlamydomspores often present ........................................ 12

7. Conidiogenous cells phialidic, with a flaring collarette; conidia with basal appendage .................................................. 4. *Haptospora* (Fig. 5)

7'. Conidiogenous cells phialidic or with solitary conidia, apparently blastic, lacking a discernable collarette; conidia lacking a basal appendage .......................................................... 8

8. Conidia adhering in regular chains .......... 8. *Paecilomyces* (Fig. 6)

8'. Conidia adhering in heads or formed singly .......................................... 9

9. Conidiophores synnematous or mononematous; conidia with a distinct, chromophilic slime layer or covered by a finely warded epispore, often somewhat fusiform ............ 6. *Hirsutella* (Fig. 7)

9'. Conidiophores mononematous; conidia thin- and smooth-walled ..10

10. Conidiogenous cells single, with only the tips protruding from the nematode; in vitro single swollen phialides supported by slender stalks .................................................. 9. *Plesiospora* (Fig. 8)

10'. Complex conidiophores appearing outside the host animal, more or less verticillate ................................................................. 11

11. Conidia with a distal adhesive surface, appearing as a wall thickening; parasites of nematodes .................................. 3. *Haptocillium* (Fig. 9)

11'. Conidia lacking an adhesive structure; parasites of rotifers ..........  ................................................................. 14. *Tolypocladium* (Fig. 10)
FIGURE 2  *Rotiferophthora globispora*, conidiophores and dictyochlamy-  
dospores arising from an infected rotifer. (From Barron, 1991a.)
12. Conidiophores erect and well differentiated (stipe usually thick-walled)......................see 10. *Pochonia suchlasporia* (Fig. 18)

12'. Conidiophores usually prostrate, sometimes also erect, but hardly differentiated from vegetative hyphae........................................13

**Figure 3**  *Drechmeria coniospora*, conidiophores and conidia arising from infected nematodes; conidia, some with developing adhesive knob; nematode with conidia attached at the cephalic and anal regions: (a) from Drechsler (1941); (b) from Barron (1977).
13. Conidiogenesis polyblastic, with conidia either on sympodially produced denticles of terminal conidiogenous cells or on densely crowded, rapidly collapsing denticles laterally along intercalary cells of prostrate fertile hyphae; dictyochlamydospores absent..............14

13’. Conidiogenesis phialidic; phialides aculeate, more or less persistent, each producing several conidia .......................................................15

**Figure 4** *Pseudomeria mucosa*, two infected rotifers with juvenile and mature conidiophores, detail of conidiophores, and conidia with mucous sheath, in those at the bottom sheath distorted after attachment. (From Barron, 1980b.)
14. Conidiiferous denticles persistent, mostly in terminal position, sometimes inserted next to discrete conidiogenous cells ..................
...............see Beauveria and Microhilum (entomogenous taxa)

14’. Conidiiferous denticles scattered along cells of fertile hyphae, soon collapsing aphanophaialides; colonies deeply woolly ..............
.................aphanocladium-like species of Lecanicillium

15. Colonies slow-growing, reaching 5−15 mm diam in 10 d; parasites of free-living nematodes or rotifers; dictyochlamydomesores often present ........................................................................................................ 16

15’. Colonies growing faster, reaching 15−40 mm diam in 10 d; growing on insects or fungi; if attacking nematodes, then parasitizing cysts or eggs; dictyochlamydosposes present or absent................................. 17

16. Parasites of bdelloid rotifers; intercalary phialides with a lateral neck normally present below terminal, flask-shaped or elongate phialides; conidia adhering in heads; a conspicuous oil globule present in each conidium; dictyochlamydosposes commonly present, often flattened ................................................................. see 12. Rotiferophthora

16’. Parasites of free-living nematodes; conidia balanoid, campanulate to cylindrical, subglobose to irregularly angular, mostly terminally adhesive (visible as a wall thickening at the upper, more or less

---

**Figure 5** Haptospora, conidiophores arising from infected rotifer and extra enlarged conidia: (a) *H. appendiculata*. (From Barron, 1991b.) (b) *H. endoparasitica*. (From Barron and Szijarto, 1982b.)
truncated end), produced in heads or short chains or both; sesquiphialides absent; dictyochlamydospore-like structures sometimes present.......................................................... 3. *Haptocillium* (Fig. 9)

17. Phialides exclusively solitary (if verticillate, conidia narrowly acerosa); dictyochlamydospores absent....13. *Simplicillium* (Fig. 11)

17'. Phialides at least partly in whorls ................................................. 18
**FIGURE 7** *Hirsutella rhossiliensis*, conidiophores and conidia, drawn with and without the slime layer. (From Minter and Brady, 1980.)

**FIGURE 8** *Plesiospora globosa*, infected nematode with conidiophores and conidia developing in agar culture. (From Drechsler, 1971.)
18. Conidia subglobose to short-ellipsoidal, sometimes short-falcate, often cyanophilic; dictyochlamydospores spherical or irregularly shaped, often present in the aerial mycelium or in the agar; mostly parasites of nematode cysts or saprotrophic, soilborne; crystals absent in the medium .................................10. Pochonia (Fig. 12)
18'. Conidia short- or long-ellipsoid to cylindrical or falcate, not conspicuously cyanophilic; chlamydomycopes or dictyochlamydospores absent (mostly entomogenous, fungicolous, or soilborne); crystals abundantly produced in the medium; sporulation with aculeate phialides predominant; denticles with blastoconidia, if present, densely scattered along the cells of fertile hyphae; on various substrata.......................................................... 7. Lecanicillium (Fig. 13)

[If conidia 2-celled, see 1. “Cephalosporiopsis” carnivora (Fig. 14)]

8. THE GENERA
8.1. Cephalosporiopsis Peyronel, Mem. R. Accad. Sci. Torino, Ser. 2, 66:52, 1916 (Fig. 3)

Type species: Cephalosporiopsis alpina Peyronel.

This genus is not generally recognized because the identity of the type species is doubtful and species assigned to the genus are extremely heterogeneous. The best-known species that was often referred to the genus is now recognized as Plectosporium tabacinum (van Beyma) Palm et al., anamorph of Plectosphaerella cucumbera (Lindf.) W. Gams in the Phyllachoraceae (Palm et al., 1995) (syn. Cephalosporiopsis imperfecta C. Moreau & M. Moreau).

Cephalosporiopsis carnivora Drechsler (1969) was observed on free-living Rhabditis sp., but is not available in culture for further study. It is characterized by discrete, short flask-shaped phialides in moderately verticillate, generally erect conidiophores and elongate-ellipsoid or somewhat obovoid conidia, mostly divided by a cross-wall at a slight median constriction, mostly 3.0–4.2 × 1.7–2.2 μm. Frequently 1 or 2 conidia were seen adhering to the forward profile of an actively motile eelworm. This statement suggests an adhesive mechanism that would place the species near Haptocillium.

**Figure 9** Haptocillium balanoides, infected specimen of Acrobeloides buetschlii with outgrowing hyphae and conidiophores, detail of conidia attached to the head region, and three extra enlarged conidia. (From Drechsler, 1941.)

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8.2. *Drechmeria* W. Gams & H.-B. Jansson, *Mycotaxon* 22:33, 1985 (Fig. 3)

*Type species:* *Drechmeria coniospora* (Drechsler) W. Gams & H.-B. Jansson.

*Diagnosis:* Colonies very slow-growing; mycelium hyaline. Fertile hyphae erect, bearing one terminal and 2–7 intercalary phialides, the intercalary ones bearing acropleurogenous conidiiferous pegs below the upper septum, forming several conidia in basipetal succession, which form stellate clusters. Conidia of *D. coniospora* are conical, with rounded base.
and almost pointed tip, one-celled, hyaline, smooth-walled, 4–7 × 1.8–2.5 μm.

Second species: *D. harposporioides* (Barron & Szijarto) W. Gams & H.-B. Jansson (*Meria harposporioides* Barron & Szijarto, 1982a), a parasite of sessile ciliated protozoans, which differs from *D. coniospora* by having falcate conidia, 7.5–12 × 1.7–2.0 μm.

Conidiogenesis and penetration of nematodes were examined with light- and electron-microscopic methods (Jansson et al., 1984; in more detail, Dijksterhuis et al., 1991). The nematodes survived about 24 h after infection. The fungus can produce 5000–10,000 conidia at the expense of a single nematode. The formation of adhesive knobs on the tip of the conidia is an autonomous process in *Drechmeria coniospora* at the end of conidial maturation (van den Boogert et al., 1992). This adhesive knob consists of radiating fibrils visible in TEM (Saikawa, 1982a), with best resolution in KMnO₄-fixed material (Saikawa, 1982b). After attachment, an infection vesicle is formed between the cuticle layers (TEM by Dijksterhuis et al., 1990; Sjollema et al., 1993). Collagenase production is induced before penetration of the cuticle (Jansson et al., 1985a). Hyphae penetrate the nematode via the pseudocoel, without attacking internal organs.
**FIGURE 12** *Pochonia chlamydosporia* var. *catenulata*; left, several conidiophores with catenate conidia and a few extra enlarged conidia; right, development of dictyochlamydospores. (From Barron and Onions, 1966.)

**FIGURE 13** *Lecanicillium lecanii*, conidiophores and conidia. (From Gams and Zare, 2001.)
Trophic hyphae contain numerous lipid droplets, often associated with microbodies (Dijksterhuis et al., 1991). Nematodes with attached conidia were seen in animals recovered from soil (cryo-SEM by Jansson et al., 2000).

Conidia can be ingested by nematodes but do not germinate in the intestine (Jansson, 1994); direct penetration from adhering conidia is thus the only mechanism of infection. The fungus attracts susceptible nematodes (Jansson, 1982a, 1982b). Based on observation of a limited number of potential hosts (Dürschner, 1983), the conidia were seen to adhere specifically in the mouth region, where chemoreceptors are situated (Jansson and Nordbring-Hertz, 1983), in male nematodes also in the anal region of certain species. Infected animals are disturbed and are no longer attracted by colonies of the fungus. In more extended studies, this localized mode of adhesion was found in bacteriophagous, a few plant-parasitic (Meloidogyne and Aphelenchus), and animal-parasitic nematodes, while other plant-parasitic nematodes (Pratylenchus, Ditylenchus and Cricone-mella) became infected at any point, but were then not equally strongly

FIGURE 14  *Cephalosporiopsis carnivora*, infected specimens of *Rhabditis* sp. with outgrowing conidiophores developing on an agar plate, and conidia, some extra enlarged. (From Drechsler, 1969.)

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parasitized (Jansson et al., 1987). Insect-parasitic species of *Neoapectana* and *Heterorhabditis* occasionally showed adhesion of conidia but normally no further penetration (Poinar and Jansson, 1986), while plant-parasitic species of *Rhabditis* were susceptible. On *Acrobeloides* conidia also adhered but without subsequent penetration (Dijksterhuis et al., 1993). The observations on nematode attraction and conidial adhesion were reviewed by Dackman et al. (1992). In several studies before 1990, sialic acid (acetyl-neuraminic acid), localized in the head and tail regions, was assumed to bind to a lectin located on the parasite’s conidia. Sialic acid was the only one of 21 carbohydrates tested that inhibited adhesion. Limulin, a lectin specifically binding sialic acid, reduced attraction to the fungus and adhesion of conidia (Jansson and Nordbring-Hertz, 1983, 1984). A carbohydrate receptor for chemotaxis with sialyl- and mannosyl-related residues was demonstrated in *Caenorhabditis elegans* and *Panagrellus redivivus*. In support of this hypothesis, adhesion was shown to be inhibited by flooding conidia with sialic acid or by treating nematodes with sialidase, a treatment that also reduced attraction of nematodes to the fungus (Jansson and Nordbring-Hertz, 1983, 1984; Jansson et al., 1985a, 1987). However, no lectin could be isolated from the conidia and no sialic acid residues were directly demonstrated in the cephalic region of *P. redivivus*. Other compounds mimicking sialic acid were then suggested as mediators of the effect. Surface receptors are still unknown in the host, contrasting with those involved in the lectin-mediated contact between nematode-capturing *Arthrobotrys* species and their prey (review by Tunlid et al., 1992). Pronase treatment of the nematodes also prevents adhesion of these conidia to *Caenorhabditis elegans*, but the nematodes regenerate the lost protein material after 2 h in Tris buffer (Jansson, 1994). In the same paper, the adhesion is suggested to be mediated by sensilla exudates. The fibrillar layer of the adhesive knob is not altered during adhesion. This layer is dissolved in Pronase E. The protease inhibitor chymostatin inhibits infection, suggesting involvement of chymotrypsin-like proteases in the infection process (Jansson and Friman, 1999). Motile nematodes infected with conidia of *D. coniospora* can also be caught by a second nematophagous fungus. Dijksterhuis et al. (1994) found that hyphal penetration by *Arthrobotrys oligospora* as a secondary invader is inhibited and its hyphae are often dead in proximity to those of *D. coniospora*. Populations of *Meloidogyne incognita* were reduced in 250-cm³ pots with sterile or unsterile soil by adding suspensions of 10⁸ conidia per pot or 1000 living, infected *Panagrellus redivivus* as carriers (Jansson et al., 1985b). Population densities of *D. coniospora* in soil can be enhanced by increasing the organic matter content (by application of lucerne meal or a barley root system), which first stimulates bacteriophagous nematodes and, secondarily, the population of the fungus (van den Boogert et al., 1994). In field soils, no positive rhizosphere effect was observed. Application of this fungus for biological control is not considered feasible because of its narrow host spectrum and because phytophagous nematodes are not intensely parasitized.
8.3. *Haptocillium W. Gams & Zare, Nova Hedwigia 72:334, 2001* (Fig. 9)

*Type species:* *Haptocillium balanoides* (Drechsler) Zare & W. Gams (*Verticillium balanoides* (Drechsler) Dowsett et al., 1982).

*Diagnosis:* Colonies slow-growing, reaching 5–15 mm diam on PDA after 10 days. Conidiophores erect or prostrate, bearing verticillate or solitary phialides which are more or less swollen near the base. Conidia balanoid, campanulate to cylindrical, subglobose to irregularly angular, mostly terminally adhesive, produced in heads or short chains or both. Dictyochlamydospores sometimes present. Mostly parasites of free-living nematodes, to which the conidia become attached by means of an adhesive apical layer. A detailed account of the genus is given by Zare and Gams (2001b).

Gams (1988) synonymized several taxa described by Drechsler under *V. balanoides*. Molecular studies have shown, however, that more species are to be distinguished.

8.3.1. Key to the Species (modified after Zare and Gams, 2001b)

1. Conidia at least partly in chains, sometimes in heads ....................2
1’. Conidia always in heads, never in chains ....................................3
2. Conidia slightly irregularly angular, small, 1.3–2.0×1.0–1.7 μm, in heads and short chains .................................................................

   *H. sinense* (K. G. Zhang, L. Cao & Z. Q. Liang) Zare & W. Gams
   [If conidia globose, 1.3–1.8 μm diam, then “*Verticillium* cocci- sporum* (Drechsler) W. Gams]

2’. Conidia of two types; campanulate to cylindrical, 2.7–3.0×1.3–1.5 μm, in heads, and globose to subglobose, 4.2–4.5×3–3.5 μm, often catenate ..........*H. campanulatum* (Glockling) Zare & W. Gams

3. Conidia globose with mostly 5 apical adhesive buds, 3.5–5.0×2.2–2.5 μm.........“*Verticillium* coronatum* Barron (1989)
3’. Conidia cylindrical .................................................................4
3′. Conidia balanoid to elongate balanoid ........................................5
4. Conidia cylindrical to campanulate, 2.7–3×1.3–1.5 μm.................

   .................................................................see *H. campanulatum*
4’. Conidia cylindrical, 3.8–4.0 × 1.3–1.5 μm ..............................................................H. rhabdosporum Zare & W. Gams
5. Conidia triangular to elongate-balanoid, with inconspicuous terminal wall thickening, measuring 3.0–4.0 × 1.7–2.0 μm; elongate conidia absent.................................H. zeosporum (Drechsler) Zare & W. Gams
5’. Balanoid conidia mostly with pronounced terminal wall-thickening, elongate conidia scarce or frequent.................................................................6
6. Balanoid to triangular conidia mostly 2.5–3.0 × 1.5–2.0 μm; elongate conidia (5.0–7.5 × 1.5–2.0 μm) frequent; phialides slender, aculeate on OA; polyphialides absent..........................................................H. balanoides (Drechsler) Zare & W. Gams
6’. Balanoid to subglobose conidia 2.2–3.2 × 1.3–2.3 μm, elongate conidia scarce (if present); phialides slender or swollen on OA; polyphialides usually absent...........H. sphaerosporum (J.B. Goodey) Zare & W. Gams (Fig. 15)
6’’. Balanoid conidia 3.2–3.6 × 2.5–3.0 μm, elongate conidia rather frequent, 5.7–6.5 × 2.0–2.2 μm; polyphialides frequent .........................H. glocklingiae Zare & W. Gams

Because of the lectotypification of *H. balanoides* by CBS 250.82 (Gams, 1988), an isolate that takes a somewhat isolated position among those available, most of the isolates commonly identified as that species now have to be called *H. sphaerosporum* (Fig. 15), and most of the information compiled below is likely to refer to that species.

### 8.3.2. Ecology and Application

The conidia of *H. balanoides* attract nematodes (Jansson, 1982a, 1982b). Drechsler (1941) originally described *Cephalosporium balanoides* from *Plectus parvus* and *Acrobeloides buetschlii*. The species was then reported as an endoparasite of *Rhabditis terricola* from various soils in Ontario (Barron, 1978), in nematodes in mosses in a birchwood in England (Duddington, 1951), and from deciduous and coniferous leaf litter, old dung, and coastal vegetation in Ireland (Gray and Duff, 1982; Gray, 1983c). *Haptocillium balanoides* occurred in 50% of agricultural soil samples taken in Westfalen, Germany (Dürschner, 1983). It was also commonly encountered in the maritime Antarctic (Gray et al., 1982; Gray and Lewis Smith, 1984). The species was regularly found on nematodes in Manitoba soils (Dowsett et al., 1982). The host range included all species of the Rhabditida, Aphelenchida, and Tylenchida tested, but not the Dorylaimida (Dürschner, 1983). The fungus seems to recognize suitable host nematodes (Dürschner-Pelz and Atkinson, 1988). The temperature minimum for growth was about 15°C (Gams, 1988). *Haptocillium balanoides* (sensu lato) was frequently observed in nematodes associated with needles of decaying Japanese red pine
trees (Watanabe, 2000); it has also been reported from nematodes in various soils of El Salvador (Búcaro, 1983). An over-winter decline in populations of the stem nematode *Ditylenchus dipsaci* was ascribed to infections by *H. balanoides* (Atkinson and Dürschner-Pelz, 1995). Conidial production was assessed on different host nematodes: the most frequent host, the stem nematode *Ditylenchus dipsaci*, yields about 15,000 conidia per cadaver, *Globodera rostochiensis* about 11,600, and *Panagrellus redivivus* about 840. This could mean that the association with phytophagous nematodes is advantageous to the parasite, while bacteriophagous nematodes may attract too many other microbes that antagonize the endoparasite (Atkinson and Dürschner-Pelz, 1995).

**Figure 15** *Haptocillium sphaerosporum*, conidiophores and conidia from agar cultures of several isolates. (From Zare and Gams, 2001b.)
The great differentiation of species recently observed in this genus will necessitate detailed ecological studies with different isolates in order to select efficient antagonists and to determine which are the most promising for biological control in a particular soil (Hay, 1995; Hay and Regnault, 1995).

8.4. **Haptospora** Barron, *Can. J. Bot.* 69:503, 1991 (Fig. 5)

*Type species:* *Haptospora appendiculata* Barron.

*Diagnosis:* Conidiophores simple or slightly branched; conidiogenous cells (phialides) flask-shaped, solitary or in clusters, with a membranous collarette; intercalary phialides occasionally present. Conidia one-celled, hyaline, with a basal appendage. Parasitizing bdelloid rotifers, forming a mycelium of swollen cells.

8.4.1. **Key to the Species**

1. Conidia globose, 2.5–3.2 μm diam .................................................................

   ...................... *H. endoparasitica* (Barron & Szijarto) Barron

1'. Conidia bilobed ........................................................................................................ 2

2. Conidia T-shaped, 2.8–3.2 μm long, 2.8–3.2 μm broad ....................

   ........................................... *H. tribrachispora* (Barron & Szijarto) Barron

2'. Conidia Y-shaped, 5.5–6.5 μm long, 5.0–7.0 μm broad .....................

   .................................................................................................. *H. appendiculata* Barron

As in *Rotiferophthora*, conidia of *Haptospora* are ingested and germinate from the mastax (Barron, 1991b).


*Type species:* *Harposporium anguillulae* Lohde.

*Diagnosis:* Colonies slow-growing, whitish. Vegetative hyphae tending to be broad and thick-walled, particularly when submerged in the agar. Fertile hyphae with elongate aggregates or clusters of phialides. Phialides mostly consisting of an almost globose venter and one to several narrow conidioferous necks. Conidia adhering in heads (in one species, in chains), in most species gracefully bent, in others cylindrical or more irregularly shaped. Most species of the genus parasitize nematodes, but some recently described ones are found in bdelloid rotifers.

*Synanamorphs:* Arthroconidia or blastoconidia of *Hirsutella* (Glockling, 1998b; Hodge et al., 1997).
Teleomorph of *H. anguillulae*: *Atricordyceps harposporifera* Samuels, New Zealand J. Bot. 21:171, 1983 (found on an arthropod [millipede?]).

Classical descriptions: Zopf (1888) gave a detailed description, establishing its classification among the hyphomycetes, and a first description of intercalary chlamydospores; Karling (1938) frequently found the species in the U.S.A. and gave an extended description of the development of conidiophores and conidia.

8.5.1. Key to the Species

(Measurements are given unaltered from the original diagnoses; some authors give the total length, others the straight line spanning the curved conidia as length. Species parasitizing nematodes unless otherwise stated.)

1. Conidia bent in circles or spirals..........................................................2
1’. Conidia of other shapes.................................................................16

2. Conidia with an almost straight base and bent distal part...............3
2’. Conidia consistently bent throughout.........................................4

3. Conidia with a circular bend in the upper part, 15–25 (as a straight line) × 0.7–1.0 μm, with a terminal 2–5-μm-long point filled with wall material, and with a viscid drop at the base..........

   ........................................................................................................... *H. oxycoracum* Drechsler

3’. Conidia with a rather sharp bend above the middle, base 14–17 μm, distal arm almost straight, 9–11 μm, broadest part 2.2 μm..............................................*H. angulare* Barron

4. Conidia bent in one direction..............................................................5
4’. Conidial bends changing direction................................................11

5. Conidia forming about half a circle.................................................6
5’. Conidia forming less than half a circle.........................................9
5”’. Conidia forming about three-fourths of a circle or ellipse.........10

6. Besides helicoid conidia blastoconidia and/or arthroconidia commonly produced.................................................................7
6’. Secondary conidia unknown or all conidia very small ...............8

7. Conidia 7–16 (straight line, total length 12.5–35 μm) × 0.7–2.5 μm; arthroconidia conspicuous, cylindrical, 9.5–11.5 × 3–4 μm.............................................*H. arthrosporum* Barron

7’. Circular conidia 9–16 (total length) × 1.0–2.0 μm, obovoid blastoconidia 3.0–6.5 × 1.5–2.0 μm, arthroconidia 14–17 × 4 μm.........................................................*H. janus* Shimazu & Glockling

8. Conidia 13–17 μm long (7–13 μm when measured in a straight line), 1–2 μm wide .................................................................*H. anguillulae* Lohde
8'. Conidia smaller, 4.5–9 (straight line?) × 1–1.5 μm; arthroconidia present ................................................................. H. lilliputanum M. S. Dixon
9. Conidia curved in ascending spiral, distally hardly pointed, 7.0–(9.5)–14.0 (straight line?) × 0.6–0.9 μm .......................................................... H. microspirale X. Z. Liu et al.
9'. Conidia crescent-shaped, distal point filled with wall material, 10–16 (straight line) × 2.5–3.0 μm ...................... H. microsporum Glockling
(if on rotifers, see Rotiferophthora torquatispora)
10. Conidia filamentous, bent to enclose three-fourths of an ellipse, total length 18–50 μm; arthroconidia with pointed end filled with wall material for 1.5–2 μm, total length 20–30 × 1.3–1.9 μm ................................................................. H. cycloides Drechsler
11. Conidia filiform, undulate, spirally bent, total length 8–12 × 0.5–0.8 μm ................................................................. H. leptospira Drechsler
11'. Conidia broader ...................................................................................................................... 12
12. Conidia twisted in the middle, appearing V-shaped, 5–11 × 0.7–1.5 μm, additional clavate phialoconidia with rounded base, 12–15 × 3–4 μm ................................................................. H. drechsleri Barron
12'. Change of direction more gradual ................................................................................... 13
13. Spiral conidia 12.5–20.5 × 1.2–2.0 μm, with basal slime drop; synanamorph of broadly fusiform hirsutella-like blastoconidia, 5.6–8.7 × 1.8–3.1 μm, abundantly produced; arthroconidia 11.0–23.5 × 3.2–4.7 μm ............................................................. H. cerberi W. Gams et al.
13'. Blastoconidial synanamorph absent ............................................................................. 14
14. Conidia with both ends pointed, 6.0–8.0 × 1.5–2.0 μm ................................................................. H. spirosporum Barron
14'. Conidia up to 1.7 μm wide, not sharply pointed ............................................................ 15
15. Conidia with constant width over most of the length, 20–48 × 0.5–1.3(–1.7) μm, with mucous base ......... H. helicoides Drechsler
15'. Conidia with pronouncedly wider distal third, 25–33 × 1.3–1.7 μm ...................... H. cocleatum Drechsler
16. Conidia straight for the major part .............................................................................. 17
16'. Conidia of more irregular shapes ................................................................................. 22
17. Conidia regularly cylindrical, mostly straight ................................................................. 18
17'. Conidia of other shapes ..................................................................................................... 21
18. On rotifers; conidia cylindrical, slightly bent, forming irregular chains, 9–11 × 1.7–2.5 μm ............................................................. H. botuliforme Barron
18'. On nematodes; conidia mostly straight, adhering in heads ...................................... 19
19. Conidia narrowly cylindrical, straight, 6–9 × 0.3–0.5 μm ............................................................. H. angustisporum Monoson & Pikul
19'. Conidia mostly exceeding 1 μm in width ................................................................. 20
20. Conidia cylindrical, straight, 2.5–5.0 × 0.7–1.5 μm.......................... H. baculiforme Drechsler

20'. Conidia long cylindrical with rounded ends, 22–27 × 1.7–2.5 μm, mostly straight................................. H. cylindrosporum Barron

21. Conidia cylindrical to allantoid, 3–5 × 0.9–1.2 μm..............................

21'. Conidia slender obclavate, tapering distally, with bent tip and adhesive apical spur, 12–26 × 1.0–1.8 μm H. subuliforme Drechsler

22. Conidia reniform or triangular.........................................................22

22'. Conidia with a laterally displaced basal apiculus and various shapes of the distal part.................................................................24

23. On nematodes; conidia reniform, 3.2–4 μm long.................................

23'. On rotifers, conidia with bilobed apex, thus appearing regularly triangular, with rounded ends, 7.2–9.0 × 6.3–7.2 μm (up to 11 μm in culture)............................... H. trigonosporum Barron & Szijarto

(If conidia with a basal appendage, see Haptospora appendiculata)

24. Conidia rather slender, apex rounded or with a simple point...........25

24'. Conidia with bulbous base, apex with upwards directed beak or with a double beak ...........................................................................26

25. Conidial apex rounded, conidia 4.5–6.5 × 0.8–2.1 μm....................... H. bysmatosporum Drechsler

25'. Conidial apex pointed in axial direction, conidia 3.5–4.0 × 1.0–1.5 μm .......................................................... H. diceraeum Drechsler

26. Conidial apex with a sharp point in distal direction, conidia 4–5 × 4–5 μm..................................................... H. rhynchosporum Barron

26'. Conidial apex with two unequal beaks, conidia 3–9 × 2–3 μm........ H. dicorymbum Drechsler

Electron-microscopic observations were concerned with H. anguillulae (Saikawa, 1982c), H. subuliforme (Saikawa and Morikawa, 1985), and H. oxycoracum (Saikawa et al., 1983).

8.5.2. Ecology

Harposporium anguillulae is the most widely distributed species. Most reports of the genus are from temperate regions, but a few species have also been reported from El Salvador (Bicaro, 1983) and Central America (Persmark et al., 1995). The biology of H. anguillulae was studied in detail by Aschner and Kohn (1958), who showed that species of this genus could be easily grown in culture. Harposporium lilliputanum and H. cycloides were grown in culture by Glockling and Shimazu (1997). The conidia of H. anguillulae and other species are ingested orally and lodge in the esophagus of the prey, whence they colonize the body.
(see also TEM study by Saikawa et al., 1983), but *H. subuliforme* can also adhere to a nematode externally due to an adhesive apical bud, which produces an adhesive substance after contact with a nematode (Saikawa and Morikawa, 1985). Because the conidia can be ingested only by saprophagous (bacteriophagous) nematodes, species of *Harposporium* have no effect on phytophagous nematodes. The seasonality and distribution in agricultural soils were studied by Persmark et al. (1996a) as described in the general part. Living mycelium (but not conidia) of *H. anguillulae* attracts nematodes (Jansson and Nordbring-Hertz, 1979), in particular species of *Panagrellus, Ditylenchus*, and *Aphelenchoides*, but not of *Pratylenchus* (Jansson and Nordbring-Hertz, 1980).

In Brazil, infective larvae of *Haemonchus contortus* (Trichostrongylid nematode parasites of sheep) were eliminated for 99.5% by adding 300,000 conidia of *H. anguillulae* to 1 g of feces (Charles et al., 1996).


*Fig. 7*

*Type species:* *Hirsutella entomophila* Pat.

*Diagnosis:* Colonies medium-fast-growing. Sporulation synnematous or mononematous. Conidiogenous cells flask-shaped, tapering in the middle or the distal part into one or several conidiiferous necks; conidiogenesis mostly phialidic, with several conidia agglutinated in slimy heads at each opening, sometimes solitary conidia produced with apparently blastic conidiogenesis. Conidia mostly somewhat fusiform, hyaline, smooth-walled, surrounded by adhesive slime, sometimes globose and roughened.

*Synanamorphs:* *Harposporium* (Glockling, 1998b; Hodge et al., 1997).

Most species of the genus are entomogenous, and many are synnematous. No comprehensive revision of the species has so far been published. A common nematode parasite is the mononematous *H. rhossiliensis* Minter & B. L. Brady (Minter and Brady, 1980), a name that predates the synonymous *H. heteroderae* Sturhan & R. Schneider 1980 by a few months. Phialides solitary, 18–33×3–5 μm, tapering to 0.5–0.7 μm; conidia formed singly or in pairs on phialides, ellipsoid with a more or less apiculate base and a voluminous persistent slime layer, 7–11×4.8–7.5 μm (measured including the slime).

8.6.1. **Ecology of *H. rhossiliensis***

The species has been isolated from *Criconemella xenoplax, Heterodera avenae, Meloidogyne javanica*, and, as *H. heteroderae*, from *Heterodera humuli* (Sturhan and Schneider, 1980). It successfully infected several other species of *Heterodera, Ditylenchus destructor, Meloidogyne hapla,*
Pratylenchus penetrans, Anaplectus granulosus, and even larvae of Globodera rostochiensis, but not members of the Tylenchidae (Sturhan and Schneider, 1980). A Petri dish population of Ditylenchus dipsaci is killed in vitro in 4 days, and one of M. incognita juveniles in 2 days (Cayrol et al., 1986). Conidia adhere to the nematode body and penetrate it; in Acrobeloides a preferential adhesion in the head and tail regions was observed (Venette et al., 1997). Hirsutella rhossiliensis is considered responsible for rapid fluctuations of C. xenoplax populations in peach orchards (Stirling, 1988, 1991; Zehr, 1985). Without nematodes, the population of H. rhossiliensis in soil dies out (Jaffee, 1991). In legume rhizospheres the population of the bacteriophagous nematodes of the genus Acrobeloides increased, followed by an increase in the population of H. rhossiliensis, but these nematodes were much less vigorously attacked by the fungus than were populations of Heterodera schachtii (Venette et al., 1997).

Densities of H. rhossiliensis in a Swedish agricultural soil peaked in September–November (Persmark et al., 1996). The population in the soil follows that of its host, Heterodera glycines in soybean fields, with midseason maxima, declining in alternating maize crops (Chen and Reese, 1999).

The conidia of the fungus are infective only as long as they are attached to the phialide (McInnis and Jaffee, 1989), and therefore the species seems less suited for biological control than species of Haptocillium (Hay and Bateson, 1997). Moreover, conidial germination can be greatly affected by soil fungistasis (Jaffee and Zehr, 1985; Stirling, 1988). The fungus exerted little population control unless host densities were high (Kerry and Jaffee, 1997). On an agar pH gradient, no growth was observed below pH 5 (López-Llorca et al., 1994). Isolates obtained from different nematodes and origins showed considerable genetic variation but had uniform characters of nematode pathogenicity; only isolates originating from Hoplolaimidae grew more slowly, had larger conidia, and were less pathogenic toward nematodes than isolates from other hosts (Tedford et al., 1994). When growing on J2 larvae of Meloidogyne hapla, the fungus produced 78–124 conidia from a single individual. Addition of 1.9 vegetative colonies/cm³ soil caused a 50% decrease in J2 penetration of lettuce roots, while lettuce weight, root galling, or egg production were not affected (Viaene and Abawi, 2000). When applied in combination with Pochonia chlamydospora, H. rhossiliensis could not be detected from lettuce roots and control was not improved by the combination; an inundative release of the fungus would be necessary at every lettuce planting, as the fungus did not survive over long periods in the soil (Viaene and Abawi, 2000). Patel et al. (2001) attempted production of inoculum in liquid culture.

A second nematophagous species, Hirsutella minnesotensis S. Chen et al., was found as a pathogen of second-stage juveniles of the soybean cyst nematode, Heterodera glycines (Chen et al., 2000). This species is morphologically very similar to the mite parasite H. thompsonii F. E. Fisher, with equally
roughened globose solitary conidia, but it has a more strongly swollen base of the conidiogenous cells and larger conidia, 4–6 \( \mu \)m in diameter. Other, entomogenous species of *Hirsutella* did not attach to nematodes with their conidia and had no controlling effect (Cayrol et al., 1986).


*Type species:* *Lecanicillium lecanii* (Zimm.) Zare & W. Gams.

*Diagnosis:* Colonies rather fast-growing, reaching 15–30 mm diam in 10 d at 20°C on PDA or MEA, white or yellowish. Conidiophores little differentiated from the subtending hyphae, commonly arising from aerial hyphae, initially erect with one or two whorls of phialides, then usually prostrate and bearing large numbers of phialide whorls or single phialides. Phialides aculeate, bearing often fasciculate groups of conidia, often positioned at a right angle with the phialide tips, in some taxa forming chains; some taxa also forming short, basally swollen, rapidly collapsing “aphanophialides” which bear single conidia. Conidia short- to long-ellipsoid to falcate with pointed ends. Chlamydospores, dictyochlamydospores, or swollen hyphal portions absent. Octahedral (sometimes also prismatic) crystals commonly present in the agar medium. Most species are entomogenous or fungicolous (Zare and Gams, 2001a).

*Teleomorphs:* *Torrubiella*, *Cordyceps*

*Lecanicillium psalliotae* (Treschow) Zare & W. Gams (once found in a cyst of *Globodera rostochiensis*) and “*Verticillium* leptobactrum” W. Gams (mainly in *Heterodera* eggs) were occasionally isolated from nematodes (Gams, 1988). Godoy et al. (1982) and Gintis et al. (1983) mentioned the rare observation of *V. lecanii* (Zimm.) Viégas [probably now *L. muscarium* (Petch) Zare & W. Gams (Fig. 16a)], *V. lamellicola* (F. E. V. Smith) W. Gams (Fig. 19a), and *V. leptobactrum* (Fig. 19b) as parasites of cysts and eggs of *Heterodera* and *Meloidogyne* species. For the latter two species see *Simplicillium*. Uziel and Sikora (1992) deliberately applied isolates of “*Verticillium lecanii*” originating from insects to control cyst nematodes, *Globodera pallida*, under artificial conditions in water agar cultures. Among 14 isolates tested, 8 [most of them probably *L. muscarium* and one *L. longisporum* (Petch) Zare & W. Gams (Fig. 16b)] successfully parasitized eggs after 2 months. Meyer and Wergin (1998) observed colonization of cysts and females of soybean cyst nematodes when *L. lecanii* was added to monoxenic nematode cultures, with fungal multiplication in the gelatinous matrix but relatively little penetration of the eggs. Observed antagonistic effects by this fungus reducing viability of cyst nematode eggs (Hänsler, 1990) might be attributed to chemical action.

*Type species:* *P. variotii* Bain.

*Diagnosis:* Colonies medium-fast-growing, powdery due to long chains of dry conidia. Conidiophores erect, with terminal and intercalary whorls of phialides. Phialides consisting of a swollen venter that tapers strongly in the upper part into a slender conidiiferous neck. Conidia one-celled, hyaline, dry, adhering in long chains.
The type species of the genus belongs to the Eurotiales. Several members of Clavicipitaceae have been included in the genus (Samson, 1974). The best known of these, *P. farinosus* (Holm : Fr.) A. H. S. Brown & G. Smith, teleom. *Cordyceps memorabilis* Ces., is a common entomogenous species, for which the genus *Isaria* Pers. : Fr. is being reintroduced (K. T. Hodge et al., in preparation). *Paecilomyces lilacinus* (Thom) Samson (Fig. 6) also belongs to the Clavicipitaceae, although it is unrelated to *P. farinosus* (Sung et al., 2001). This is a rather common pathogen of nematode eggs in soil (Dackman and Nordbring-Hertz, 1985; Dackman et al., 1985; Stirling, 1998, 1991). Numerous experiments have been carried out in view of its application in biological control (Kerry and Evans, 1996; Stirling, 1991). However, most experiments carried out so far with the species for biological control lacked adequate controls to ascertain the specific fungal effects (Kerry, 1989, 1990). Its potential human pathogenicity (ocular and cutaneous infections, onychomycosis, sinusitis, and deep infections in immunocompromized patients, de Hoog et al., 2000) seems to preclude its practical application, but some genetic difference was found between human-pathogenic isolates and the nematode parasites (R. A. Samson, personal communication, 2002). Different isolates vary greatly in their pathogenicity to nematodes (Stirling, 1991). The nematophagous ability of the isolates tested was somewhat correlated with their UV resistance; a similar grouping of isolates could also be achieved by means of random amplified polymorphic DNA (RAPD) (Gunasekera et al., 2000). The species was found to efficiently parasitize eggs of *Meloidogyne incognita* and *Globodera pallida* in Peru (Jatala et al., 1979). Some other sensitive nematode species were listed in a comprehensive review by Jatala (1986). The fungus not only attacks the egg shell, it also has toxic effects (Jatala, 1986). Production of the antibiotics leucinostatin and lilacin and chitinolytic enzymes have been documented (Morgan-Jones and Rodriguez-Kabana, 1988). Production of a basic serine protease is induced in *P. lilacinus* by cultivation on chitin or vitellin; this enzyme destroys eggs of *Meloidogyne hapla* and is regarded as a crucial component in pathogenesis (Bonants et al., 1995; Segers et al., 1999). Colonization of *Meloidogyne* eggs with appressorium formation was illustrated in SEM photographs by Segers et al. (1996). Increased populations of the fungus were also associated with decline of *Rotylenchulus reniformis* in tomato in India, giving a control comparable to that by carbofuran (Reddy and Khan, 1988). In some *Meloidogyne*-suppressive soils in California, the natural populations of *Pochonia chlamydosporia* and *P. lilacinus* seemed to play a minor role in regulating the nematode population (Gaspard et al., 1990a). Population densities of *P. lilacinus* were positively correlated with those of *P. chlamydosporia* in tomato field soils in California, but not with those of *Meloidogyne incognita* (Gaspard et al., 1990b). Application of 10 or 20 g of fungus-infested wheat kernels per microplot (0.76 m diam) at planting time (even better with an additional treatment 10 days before planting) gave good protection against
*Meloidogyne incognita* and increased tomato yield significantly, particularly at temperatures between 24 and 28°C (Cabanillas and Barker, 1989; Cabanillas et al., 1989). Under field conditions in Peru, application of the fungus in potato soils gave a lower galling index due to *Meloidogyne incognita* than nematicide treatments (Jatala et al., 1980), and a single application appeared sufficient to establish the fungus in the soil (Jatala et al., 1981). Selective media for monitoring the species have been devised by Mitchell et al. (1987: PDA with, per liter, 10 g NaCl, 50 mg pentachloronitrobenzene, 50 mg benomyl, 1 mL Tergitol NP10, and antibacterial antibiotics), Cabanillas and Barker (1989: PDA with dichloran and oxgall together with antibacterial antibiotics), and Gaspard et al. (1990a: chitin-rose bengal agar with 50 mg/L iprodione). Fassatiova and Lysek (1982) obtained this species and the similar *P. marquandii* (Massee) S. Hughes from eggs of *Ascaris lumbricoides* exposed in soils in the Czech Republic, Pakistan, and Cuba. *Paecilomyces lilacinus* (less than *P. marquandii*) also infected and killed eggs of the canine roundworm, *Toxocara canis*, penetrating them by means of appressoria (Basualdo et al., 2000). Therefore *P. lilacinus* might also be used as a biological control agent against animal helminths in vivo.


*Type and only species: Plesiospora globosa* Drechsler.

*Diagnosis:* Phialides produced singly, tolypocladium-like inflated, remaining inside the cuticle of the infected nematode, protruding only with the opening. Conidia globose, hyaline, smooth-walled, 1.8–2.5 μm in diameter.

The species is obviously very similar to *Tolypocladium* and *Haptocillium*, but differs from them in vitro by forming single, swollen phialides supported by slender stalks. *Plesiospora globosa* was found in nematodes in forest detritus in Wisconsin. The fungus was also observed in Canada by Barron (1978), in Apsheron, Azerbaidzhan, by Shilova (1987) and in Japan and Illinois by S. L. Glocking (personal communication, 2002).


*Type species: Pochonia humicola* Bat. & O. M. Fonseca = *P. chlamydosporia* (Goddard) Zare & W. Gams (Zare et al., 2001) (*Verticillium chlamydosporium* Goddard 1913).
**Diagnosis:** Colonies rather fast-growing (15–40 mm diam in 10 days). Conidiophores usually prostrate and little differentiated from vegetative hyphae, sometimes erect. Conidiogenous cells phialides, verticillate or solitary, aculeate. Conidia subglobose, ellipsoidal to rod-shaped, isodiametric-polyhedral, or falcate with blunt ends, adhering in globose heads. Dictyochlamydospores often produced. Crystals absent.

**Teleomorph: Cordyceps**

Barron and Onions (1966) distinguished *Diheterospora* from *Verticillium* because of the presence of dictyochlamydospores. Gams (1971, 1988) did not recognize this criterion as having generic value, but molecular studies by Zare et al. (2000) and Sung et al. (2001) showed the phylogenetic distinctness of these parasites of nematode cysts and eggs. In addition to the common presence of dictyochlamydospores or at least swollen hyphal cells, the chromophilic behavior of the conidia and the absence of crystals can be taken as morphological criteria to distinguish the genus from *Lecanicillium*. The genus was revised by Zare et al. (2001).

### 8.10.1. Key to the Species (modified from Zare et al., 2001)

1. At least part of the conidia crescent-shaped or falcate..........................
   P. bulbillosa (W. Gams) Zare & W. Gams

1'. Conidia not crescent-shaped or falcate ...........................................2

2. Conidia isodiametric-polyhedral; dictyochlamydospores present, usually on the agar surface *P. gonioides* (Drechsler) Zare & W. Gams

2'. Conidia rod-shaped, smooth, with truncate ends, 2.0–2.5 × 0.8–1.0 μm; dictyochlamydospores sparse, submerged in the agar; so far known only from rotifers in pine litter in Japan..........................................
   P. microbactrospora Zare & W. Gams

2". Conidia of other shapes, oval, subglobose to subcylindrical, smooth; dictyochlamydospores above or in the agar ...........................................3

3. Dictyochlamydospores, at least in fresh isolates, abundant, particularly in the aerial mycelium; conidiophores typically prostrate ..............4

3'. Dictyochlamydospores, if present, mostly submerged in the agar; conidiophores prostrate or erect.........................................................5

4. Conidia only in heads, never in chains, (1.8–)2.5–4.5 × 1.0–1.2–2.2 μm........... P. chlamydosporia (Goddard) Zare & W. Gams
   ...................................................... var. chlamydosporia (Fig. 17)

4'. Conidia mostly in chains, (1.5–)2.0–3.5 × 1.5–3.0 μm; some heads may be present.... P. chlamydosporia var. catenulata (Kamyschko ex Barron & Onions) Zare & W. Gams (Fig. 12)
5. Colony reverse developing red shades on PDA; conidiophores prostrate, verticillate; conidia globose to subglobose, 2.5–3.5 × 2.0–3.0 µm, dictyochlamydospores scantly or absent..........................

.............................................................................................. P. rubescens Zare et al.

5'. Colony reverse yellow to cream (not red) on PDA; conidiophores partly erect, richly verticillate; dictyochlamydospores partly submerged in the agar..........................

6. Conidia only in heads, never in chains, measuring 2.3–4.0 × 1.5–2.5 µm ......................... P. suchlasporia (W. Gams & Dackman)

Zare & W. Gams var. suchlasporia (Fig. 18)

6'. Conidia mostly in chains, some heads may be present, measuring 2.0–3.7 × 1.7–2.3 µm ......................... P. suchlasporia var. catenata (W. Gams & Dackman) Zare & W. Gams

Most studies deal with P. chlamydosporia var. chlamydosporia (Fig. 17).
Its teleomorph, Cordyceps chlamydosporia H. C. Evans (in Zare et al., 2001), has been found on slug eggs in tropical countries. Diheterospora catenulata Kamyschko ex Barron & Onions, which differs from P. chlamydosporia var.
chlamydosporia only by catenate phialoconidia, was relegated to varietal rank by Gams (1988), and this position is confirmed by Zare et al. (2001), in contrast to different statements by Carder et al. (1993). A similar, so far unnamed, teleomorph was found for this variety by Evans on a beetle larva in Ecuador.

A second common species in central and northern Europe is P. suchlasporia (Fig. 18), which has more richly verticillate, erect conidiophores and mostly submerged dictyochlamydospores. Even after P. suchlasporia was segregated from the V. chlamydosporium complex, P. chlamydosporia is found to be rather heterogeneous in molecular analyses and in their ecological qualities (Arora et al., 1996; Kerry et al., 1986, 1993). This species is not very closely related to the remaining taxa of the genus (Sung et al., 2001).

**FIGURE 18** Pochonia suchlasporia var. suchlasporia, conidiophores and conidia and dictyochlamydospores. (From Zare et al., 2001.)

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Pochonia gonioides was originally observed by Drechsler (1942) on a species of Bunonema, but Drechsler could not establish the mode of entry into the nematode. Recently only two isolates of this species have been available, and these were originally not directly associated with nematodes. Pochonia bulbillosa is commonly isolated in conifer soils but its association with nematodes is not ascertained, apart from its isolation as an ovicidal species from Ascaris eggs in Pakistan and Afghanistan by Fassatiová and Lysek (1982).

8.10.2. Ecology and Application

P. chlamydosporia is the species most frequently cited in studies on parasites of nematode cysts. The species has many times been associated with nematode cysts of various Heterodera species (summarized by Stirling, 1988, 1991), and it is the major egg pathogen of Heterodera species in all European and American countries examined (Bursnall and Tribe, 1974; Stirling, 1988, 1991). López-Llorca and Duncan (1988) illustrated the colonization of Heterodera avenae by species of Pochonia using SEM. The species is also found as an efficient parasite of Meloidogyne root-knot nematodes (Godoy et al., 1981; Kerry, 2001; Morgan-Jones et al., 1983; Morgan-Jones and Rodríguez-Kábana, 1988). In peanut fields, Meloidogyne arenaria was more frequently parasitized than Heterodera glycines (Morgan-Jones et al., 1981). The fungus usually does not attack potato cyst eelworms of the genus Globodera (Kerry and Crumpp, 1977). Barron and Onions (1966) and Tribe (1977) found the species also on slug eggs. Its ecology has been repeatedly studied (Juhl, 1982; Dackman and Nordbring-Hertz, 1985; Dackman et al., 1989, 1992; Gaspard et al., 1990a, b; Kerry et al., 1993). Quantification of diseased eggs of Heterodera species was described by Kerry and Crump (1977).

Media for the selective isolation and quantification of P. chlamydosporia were devised by Gaspard et al. (1990a, b): a chitin-rose bengal agar with 50 mg/L benomyl); de Leij and Kerry (1991) and Kerry et al. (1993) recommend cornmeal agar (Oxoid) with 37.5 mg carbendazim, 37.5 mg thiabendazole, 75 mg rose bengal, 17.5 mg NaCl, 3 mL Triton X-100, and antibacterial antibiotics. Crump and Kerry (1981) extracted and enumerated the dictyochlamydospores from soil. Nicolay and Sikora (1989) quantified egg parasites by a new, standardizable technique: the cysts present in a soil are extracted and crushed, and the contents are reincorporated into the original soil sample. Parasitic activity on newly formed eggs is then assessed. Bourne et al. (1994) devised several methods to quantify the capacity of the fungus to grow in sterile and unsterile plant rhizospheres. To detect P. chlamydosporia on infected plant roots with polymerase chain reaction (PCR), Hirsch et al. (2000) developed specific primers from a cloned fragment of the β-tubulin gene.
The proteinase VCP1 of *P. chlamydosporia* was found to hydrolyze egg shell proteins of *Meloidogyne* species but not those of *Globodera* (Segers et al., 1996). The egg shells of *Globodera* are also rendered resistant by being twice as thick as those of *Meloidogyne* (López-Llorca and Robertson, 1992). *Pochonia chlamydosporia* also produces a chymoelastase-like protease which hydrolyses host nematode proteins in situ (Segers et al., 1994). One of its major classes of extracellular proteases is subtilisin-like proteins, of which one to four isoforms were found in different isolates; these enzymes digest the protein component of nematodes and are important determinants of pathogenicity (Segers et al., 1999). The enzyme is similar to that produced by *Metarhizium anisopliae* (Segers et al., 1995). The fungus is a factor in natural decline of nematode populations (Kerry et al., 1982); partial sterilization of the soil with 38% formaldehyde destroyed its population and the nematode-decline effect (Kerry and Jaffee, 1997). The fungus colonizes living and, somewhat more efficiently, dead eggs of *Heterodera*, with a preference for young stages, before the embryo development is completed (Irving and Kerry, 1986). *Pochonia chlamydosporia* is also ovicidal to the large roundworm, *Ascaris lumbricoides* (Lysek and Krajci, 1987).

First attempts to apply conidial suspensions against nematodes failed (Willcox and Tribe, 1974), but numerous subsequent trials of the potential use of *P. chlamydosporia* in biological control of *Heterodera* and *Meloidogyne* species were more successful (de Leij and Kerry, 1991; Kerry and Evans, 1996; Morgan-Jones et al., 1983; Stirling, 1991; Tribe, 1980; Kerry, 2001). Kerry (1995, 2001) reviewed the biology of *P. chlamydosporia* in view of its potential application against cyst and root-knot nematodes. The fungus proliferates in calcareous loams and organic soil in England and survives for at least 3 months after application, but isolates differed greatly in their capacity to survive and proliferate in different soils (Kerry, 1989; Kerry et al., 1993; Viaene and Abawi, 2000) and also in virulence (Irving and Kerry, 1986). Along an agar pH gradient in Petri dishes, optimal growth occurred around pH 6, and some growth even at pH 3 (López-Llorca et al., 1994). This species can affect the multiplication of cyst nematodes by multiple means, not only by egg colonization (Kerry, 1990). “Control of *H. schachtii* by different isolates was related to the proportion of young females infected but not to the numbers of cysts colonized; infection resulted in few eggs being produced and many of those were parasitized” (Kerry, 1990). In peaty sand a better establishment was observed than in loamy sand or sand in tomato plots with *M. incognita*; but in microplots with sandy loam a 90% control of *M. hapla* could be achieved, provided the temperature did not exceed 25°C (de Leij et al., 1993). The species was found in 13 of 20 Californian tomato field soils examined, and its densities were positively correlated with those of *Meloidogyne incognita* and *Paecilomyces lilacinus* (Gaspard et al., 1990b). Addition of *Meloidogyne* species to the soil increased the population of *P. chlamydosporia*. In tomato soils, changes in population densities of
M. incognita and P. chlamydosporia followed each other. De Leij and Kerry (1991) observed that application of dictyochlamydospores and hyphal fragments without additional food base gave the best establishment, while Kerry et al. (1993) found addition of wheat bran to alginate pellets essential for the establishment of the fungus from granular applications. Application of chlamydospores (concentrations comparable to those observed in soils naturally suppressive to cyst nematodes, about $10^3$–$10^4$ CFU/g soil) is regarded as more efficient than alginate-bran pellets (Davies et al., 1991). Addition of 5000 dictyochlamydospores/cm$^3$ of soil caused up to 43% colonization of egg masses of M. hapla, without causing any effects on lettuce weight, root galling, or egg production (Viaene and Abawi, 2000); a control could be achieved only up to a concentration of 8 eggs/cm$^3$ soil. At high galling rates no successful biological control is possible (de Leij et al., 1992). Dictyochlamydospores can be produced on a sand-barley bran mixture in amounts of $5 \times 10^6$ g medium in 3 weeks at 20°C (Kerry and Jaffee, 1997). A small inoculum of the fungus showed strongest multiplication in soil, provided additional nutrients were available (de Leij et al., 1992). In maize and tomato soils, Bourne and Kerry (1999) observed a stronger effect than in kale soils. Added dictyochlamydospores gave an acceptable control in maize, kale, and beans, but reduced nematode infestation slightly in tomato, where significant numbers of eggs remained protected from infection inside the roots. Pochonia chlamydosporia cannot colonize the root cortex, and egg masses developing inside large galls are therefore protected from fungal infection (de Leij and Kerry, 1991). The capacity of certain isolates of P. chlamydosporia to multiply in the rhizosphere of suitable host plants without adverse effects on the plant (Bourne and Kerry, 1999; Bourne et al., 1994; Davies et al., 1991; de Leij and Kerry, 1991) is particularly relevant. The understanding of the tritrophic system is important for a successful application (Bourne and Kerry, 1999). The rhizosphere of lettuce is not easily colonized by P. chlamydosporia, possibly because of competition by other microorganisms (Viaene and Abawi, 2000). Inoculation of “poor host plants” (i.e., not attractive to Heterodera species or causing only small galls) preceding a more susceptible crop may help the fungus to build up population levels and reduce nematode levels, thus improving effectiveness of nematode control before a nematode-susceptible crop is planted. The fungus “alone is unlikely to give adequate control of pest problems, but integrated with other measures, V. chlamydosporium may provide a useful additional approach to nematode management” (Davies et al., 1991; Kerry, 1995, 2001).

The application of P. chlamydosporia can be successfully combined with the nematicide Aldicarb. This compound prevents initial nematode damage, while the fungus subsequently confers a long-term protection (de Leij et al., 1993). Application of the fungus was also successfully combined with that of an arbuscular mycorrhizal symbiont, Glomus desertorum. A combined treatment
gave optimal control on tomato nursery seedlings: fewer galls, fewer egg masses, and more parasitized eggs (68%, compared with 52% with *P. chlamydosporia* alone) (Rao et al., 1997). Chopped leaves of *Azadirachta indica* (neem) more than those of *Calotropis* also acted synergistically with *P. chlamydosporia* in reducing both the nematode population and galling in tomato in pot experiments, while increasing plant growth and egg parasitism (Reddy et al., 1999).

*Pochonia suchlasporia* (Fig. 18) has a lower temperature minimum and optimum for growth and therefore certain ecological advantages over *P. chlamydosporia*. Temperature effects on the development of these species were studied by Dackman and Bååth (1989). *Pochonia suchlasporia* is the dominating species in *Heterodera* cysts in Denmark, Sweden, and the Netherlands (Juhl, 1982; Dackman and Nordbring-Hertz, 1985; Dackman et al., 1989; Gams, 1988), while *P. chlamydosporia* is more restricted to young cysts in these countries. The former species was particularly successful in colonizing eggs and showed high chitinase and protease activities (Dackman and Nordbring-Hertz, 1985; Dackman et al., 1989). The most virulent isolate of “*P. chlamydosporia*” and “*V. chlamydosporium*” tested by Irving and Kerry (1986) was also infectious at 5°C and should probably be identified as *P. suchlasporia*. The similar *P. rubescens* showed optimal growth at pH 6, but pigment production occurred mainly in the acidic range (López-Llorca et al., 1994). In vitro, this fungus (then identified as “*V. suchlasporium*”) attacked eggs of *Heterodera* and *Globodera* species, forming appressoria and penetration hyphae with an internal infection bulb as demonstrated in TEM photographs by López-Llorca and Robertson (1992).

### 8.11. *Pseudomeria* Barron, *Can. J. Bot.* 58:443, 1980 (Fig. 4)

*Type and only species: Pseudomeria mucosa* Barron.

*Diagnosis:* Conidiophores ascending from the attacked animal, unbranched, repeatedly septate; from each cell a narrow conidiogenous neck arises that develops a succession of almost beauveria-like denticles bearing single conidia; conidia globose, 3.5–4.5 μm diam, with a conspicuous basal slime pad.

Parasitizing rotifers of the genus *Adineta* (Barron, 1980b).

### 8.12. *Rotiferophthora* Barron, *Can. J. Bot.* 69:495, 1991 (Fig. 2)

*Type species:* *R. globispora* (Barron) Barron.
Diagnosis: Conidiophores verticillate or with single branches; phialides only slightly swollen, mostly aculeate; intercalary phialides* frequently present below the terminal phialide, with a slender conidiiferous neck. Conidia subglobose, ellipsoidal, clavate or curved, with a characteristic large oil drop near the apex. Dictyochlamydospores mostly conspicuously present, often composed of fewer cells than in Pochonia; cells often in a two-dimensional arrangement. Synopsis of described species: Glockling (1998a, no key).

8.12.1. Key to the Species
(Measurements are given unaltered from the protologs.)

1. Conidia (sub-)globose ................................................................. 2
1'. Conidia not globose ................................................................. 3
2. Conidia 3.3–3.6 \( \mu \text{m} \) diam ........................................... \( \ast R. \ globispora \ast \) Barron
2'. Conidia 2.0–2.2 \( \mu \text{m} \) diam ........................................... \( \ast R. \ minutispora \ast \) Glockling
2". Conidia 3–4.5 \( \times \) 2.5–3.0 \( \mu \text{m} \) see Tolyphocladium parasiticum Barron
3. Conidia with a straight longitudinal axis ........................................ 4
3'. Conidia with a curved longitudinal axis ........................................ 18
4. Conidia ovoid to almost ellipsoidal .............................................. 5
4'. Conidia ellipsoidal, cylindrical or other shapes ................................ 9
5. Conidia 3.0–4.0 \( \times \) 2.0–3.0 \( \mu \text{m} \) .......................... see Tolypocladium parasiticum Barron
5'. Conidia larger ............................................................................. 7
6. Phialides in pairs ................................................................. \( \ast R. \ amamiensis \ast \) Glockling
6'. Phialides superimposed in drechmeria-like arrangement .................
7. Conidia top-shaped, 3.5–5.0 \( \times \) 3.0–3.5 \( \mu \text{m} \) ............................ \( \ast R. \ turbinispora \ast \) (Barron) Barron (nom. inval. Art. 37.1)
7'. Conidia obovate and larger ......................................................... 8
8. Conidia 4.5–5.5 \( \times \) 2.5–3.5 \( \mu \text{m} \); dictyochlamydospores of 8–16 cells ................................................................. \( \ast R. \ ovispora \ast \) (Barron) Barron
8'. Conidia 6.0–6.5 \( \times \) 5.5–6.0 \( \mu \text{m} \); dictyochlamydospores mostly 4-celled ................................................................. \( \ast R. \ rotiferorum \ast \) (Barron) Barron

*The term aphanophialide, introduced by Gams (1971) for ephemeral structures observed in Aphanocladium and Lecanicillium (Zare and Gams, 2001a) that soon collapse to form inconspicuous denticles, is not adequate to describe the structures of Rotiferophthora. Its conidiophores are more like those of Sesquicillium W. Gams, a genus now merged with Clonostachys by Schroers (2001). No separate term seems required to describe the structure; “intercalary phialides” suffices.

†The spelling of the epithets was modified from the original, to bring the connecting vowel in accordance with Art. 60.G ICBN.

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9. Conidia ellipsoidal.................................................................10
9'. Conidia of other shapes.......................................................11
10. Conidia oval to broadly ellipsoidal, 2.7–3.0 × 2.4–2.7 μm...........
	.................................................. R. barronii Glockling
10'. Conidia ellipsoidal, 3.5–4 × 2.0–2.5 μm..............................
	.................................................. R. tagenophora (Drechsler) Barron
10''. Conidia ellipsoidal, 5.4–6.9 × 2.9–3.1 μm..........................
	.................................................. R. ellipsospora Glockling
11. Conidia cylindrical ..............................................................12
11'. Conidia of other shapes......................................................14
12. Conidia broadly cylindrical, 5.5–7.5 × 1.8–2.3 μm.................
	.................................................. R. japonica Glockling
12'. Conidia narrowly cylindrical ..............................................13
13. Conidia narrowly cylindrical, in pronounced fascicles, 7.5–11 × 2.4–3.2 μm.............. R. cylindrospora (Barron) Barron
13'. Conidia cylindrical to slightly allantoid, 6.5–8 × 1.1–1.3 μm............. R. angustispora (Barron) Barron
14. Conidia biconical with rounded ends, 6.5–7.5 × 2.2–2.5 μm...........
	.................................................. R. biconica Barron
14'. Conidia with broader distal part...........................................15
15. Conidia resembling a maize kernel......................................16
15'. Conidia more elongate......................................................17
16. Conidia 7–8 × 3.2–4.5 μm ........................................ R. zeispora† (Barron) Barron
16'. Conidia 5.0–6.5 × 2.5–3.5 μm ................ R. intermedia (Barron) Barron
17. Conidia tooth-like, clavate, slightly indented below apex, 8.4–10.8 × 2.8–3.6 μm......................... R. denticulispora Barron
17'. Conidia obovoid-clavate, tearlike, 5.0–5.5 × 1.0–1.5 μm ...........
	.................................................. R. lacrima Glockling
18. Conidia comma-shaped or broadly obovate..........................19
18'. Conidia of other shapes....................................................21
19. Conidia broadly obovate, apex curved, tapering toward a short, truncate base, 3.5–3.7 × 2.9 μm .......... R. attenuata Glockling
19'. Conidia comma-shaped....................................................20
20. Conidia 6.0–6.6 × 2.5–3.0 μm .......... R. asymmetrica (Barron) Barron
20'. Conidia 7–9 × 4.5–5.5 μm ................ R. humicola (Barron) Barron
21. Conidia bluntly kidney-shaped..........................................22
21'. Conidia of more slender curved shapes..............................23
22. Conidia 3.0–5.0 × 1.5–2.0 μm .......... R. reniformis (Barron) Barron
22'. Conidia 5–6 × 5.0–5.5 μm ..................... R. brevipes Barron
22''. Conidia 8–11 × 5–7 μm ......................... R. lunatispora† Glockling
23. Conidia with circular outline from one side and a slit on the other, 3.0–3.2 × 1.6–2.2 μm ............. R. microspora (Barron) Barron
Conidia boomerang-shaped, with one arm narrower, 3.4–4.5 × 2.0–2.5 μm ..............................................R. guttulispora† (Barron) Barron

Conidia arcuate in about a third of a circle, 6.5–8.0 (total length) × 1.0–1.5 μm ..............................................R. torquatispora† Glockling

The first species of the genus was described by Drechsler (1942) as Acrostalagmus tagenophorus from rotifers in a rich soil. Barron (1991a) is the father of most known species. He did not generally grow these fungi in pure culture but kept permanent slides from his material as types of the new species. The species are very slow-growing and do not tend to form their phialidic propagules in culture, while dictyochlamydospores are more easily obtained.

The parasitic phase is initiated by ingested conidia lodging on the wall of the alimentary system between the mouth and the mastax. Rotiferophthora species are among the most frequently recorded parasites of bdelloid rotifers. They are devastating parasites, able to wipe out entire populations of rotifers in Petri dishes in a few days (Barron, 1991a, 1991b).

8.13. Simplicillium W. Gams & Zare, Nova Hedwigia 73:39, 2001 (Figs. 11, 19)

Type species: Simplicillium lanosoniveum (van Beyma) Zare & W. Gams.

Diagnosis: Similar to Lecanicillium, but with mostly solitary phialides arising from aerial hyphae, usually prostrate and little differentiated from the subtending hyphae. Phialides discrete, aculeate and narrow, with a very narrow tip in which collarette and periclinal wall thickening are not visible. Conidia adhering in globose slimy heads or imbricate chains.

Teleomorph: Torrubiella

Species of this genus are not normally found on nematodes, but in soil, on fungi or insects. Godoy et al. (1982) reported S. lamelicola (F. E.V. Smith) Zare & W. Gams (Fig. 19a) and Verticillium leptobactrum W. Gams (Fig. 19b) (the latter species may belong to Simplicillium as suggested by its solitary phialides, but molecular study has not yet been done) from eggs of Heterodera glycines and Meloidogyne arenaria; these two species were among the most active colonizers of M. arenaria eggs (Morgan-Jones and Rodríguez-Kában, 1988). V. leptobactrum has also been reported from nematodes in Germany (Gams, 1988).

8.14. Tolypocladium W. Gams, Persoonia 6:185, 1971 (Fig. 10)

Type species: Tolypocladium inflatum W. Gams.

Diagnosis: Colonies rather slow-growing, pulvinate, cottony, white; hyphae slender, mostly 1.0–1.5 μm wide. Conidiophores scattered over
the whole colony, short, lateral, sometimes bearing dense whorls of phialides. Phialides consisting of a moderately swollen base and a threadlike, often bent, neck. Conidia adhering in heads, globose to cylindrical, hyaline, smooth-walled. Chlamydospores absent.

_Teleomorph: Cordyceps_

Most species of the genus are soil-borne or entomogenous.

In spite of different conidiogenesis, von Arx (1986) merged the genus with _Beauveria_, but physiological (Todorova et al., 1998) and phylogenetic data (Gams et al., 1998b) show that these genera are not closely related, although both are associated with teleomorphs in _Cordyceps_ (Hodge et al., 1996).

**Figure 19** (a) _Simplicillium lamellicola_, conidiophores and conidia from several isolates; (b) _Verticillium leptobactrum_, both original.
Barron (1980a, 1983) described three species from bdelloid rotifers. All have strongly inflated hyphal cells inside the prey animal. Conidia are ingested as in *Harposporium* and *Rotiferophthora*; some conidia are digested, while others germinate in the mastax (Barron, 1983). Bissett (1983) keyed out 11 species of the genus, among which two were associated with rotifers. Bissett also included *Cephalosporium balanoides* Drechsler in *Tolypocladium*; it is now classified in *Haptocillium*. A few similar nematode-parasitic species with cylindrical, straight, or slightly bent conidia are classified in *Harposporium*. They differ from other species in *Tolypocladium* by their parasitic capacities, slower growth, and a strong tendency to form swollen hyphae.

8.14.1. Key to the Rotifer-Parasitic Species

1. Conidia (sub)globose, 3.0–4.5 × 2.5–3 μm; complex stalked chlamydospores present................................................... *T. parasiticum* Barron  
1’. Conidia asymmetrical; chlamydospores unknown.............................................2  
2. Conidia shaped like a citrus slice, asymmetrically biconvex, 2.5–3.2 × 1.5–2.0 μm..........................*T. lignicola* Barron  
2’. Conidia symmetrically triangular with rounded ends, 2.5–3.2 μm diam..........................................................*T. trigonosporum* Barron

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3

Clavicipitaceous Anamorphs

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1. INTRODUCTION

Asexual states (anamorphs) play prominent roles in the life cycles of clavicipitaceous fungi. Most are presumed to be parts of the life cycles of sexual species, but relatively few have been linked to their sexual states, especially among the insect pathogenic species. This creates a problem of naming, as fungi are formally classified based on the features of their sexual (teleomorphic) states. Anamorphs are classified based on the morphology of structures associated with spore production. They are named under the aegis of Article 59 of the International Code of Botanical Nomenclature (Greuter et al., 2000), which permits the assignation of multiple names to the same organism (among extant organisms, this practice is unique to fungi). When the sexual state (teleomorph) is known, the name of that state is preferred, but in practice the names of asexual or anamorphic states are frequently used to describe the anamorph when it occurs alone.

In general, the anamorphs of clavicipitaceous fungi are hyphomycetes that bear a close resemblance to the anamorphs of other hypocrealean fungi. Their pale or sometimes brightly colored conidiogenous structures produce aeroconidia in dry chains or slimy drops, and conidiogenesis is typically phialidic or sympodial. Many exceptions to these general rules exist, and these are discussed below under individual genera. Modern studies have revealed new connections by both cultural
(Hodge et al., 1996) and molecular methods (Gernandt and Stone, 1999), and it is expected that a number of other moniliaceous hyphomycetes will be found to have connections to the Clavicipitaceae.

2. ECOLOGICAL ROLES OF ANAMORPHS

Three different life histories predominate among members of the Clavicipitaceae. In order of diminishing numbers of species, these are insect pathogenic, plant symbiotic, and mycoparasitic. These groups are discussed in more depth elsewhere in this volume. Regardless of their primary association, many members of the Clavicipitaceae exhibit associations with insects and other arthropods. These relations are most striking among the insect pathogenic members, which seldom occur in nature apart from their insect hosts. Infective conidia produced by the anamorphs can drive large epizootics and play a significant role in regulating insect populations (Price, 1987; Ewald, 1987). Insects are also widely exploited by plant symbiotic members of the Clavicipitaceae as vectors of infective spores and as bearers of gametes. Most species of Claviceps, the ergot fungi, produce Sphacelia anamorphs adapted for insect dispersal. These produce conidia in a sticky matrix of sugars (honeydew) that attracts insects (Mower and Hancock, 1975). Insects then efficiently transfer the infective conidia to new hosts, spreading the disease. Some endophytic fungi rely on insects to disperse their male gametes. Flies transfer the conidia of Neotyphodium anamorphs to fertilize nearby stromata among these heterothallic species (Bultman, 1995; Bultman et al., 1995). The mycoparasitic species alone seemingly escape reliance on insects, but this phylogenetically diverse group has been little studied in terms of dispersal and mating systems. Their often slimy conidia hint at water dispersal, or perhaps at vector relationships that have not yet been elucidated.

Variation in anamorph form has presumably arisen in response to the selective pressures associated with effective dispersal and infection. A role for conidia as gametes has not been detected among the insect and fungus parasites. As in other fungi, conidia that are dispersed by water tend to be produced in slime; those that use insects as vectors are similarly slimy, hydrophilic, and may further possess conidiomata that increase the likelihood of contact between arthropods and spores (Ingold, 1978). The mealybug pathogen Hirsutella cryptosclerotium, for example, is effectively dispersed by rain (Fernández-García and Fitt, 1993). Wind-dispersed conidia, on the other hand, are typically dry and strongly hydrophobic.

Most often, conidia are capable of infecting their hosts, so factors affecting their production and dispersal have important impacts on the management of plant disease, and on insect populations (Hajek and St. Leger, 1994). Clavicipitaceous fungi can have significant effects on host populations. Anamorphic fungi may cycle rapidly on their hosts, producing large quantities of new inoculum in
a relatively short period of time. The relative roles that anamorphs play in life cycles vary tremendously within the group. *Cordyceps militaris*, for example, is seldom collected or isolated in its anamorphic state, although it grows well in axenic culture. In *C. militaris* it appears to be the ascospores of the teleomorph that are most important in dispersal and infection. This paradigm probably applies to many other *Cordyceps* anamorphs that have not been recorded from nature, including the *Hirsutella* anamorphs of *C. khaoyaiensis* and *C. pseudomilitaris* (Hywel-Jones, 1994), and *Paecilomyces cinnamomeus* and an Acremonium-like synanamorph produced by *Torrubiella luteorostrata* in culture (Hywel-Jones, 1993). These anamorph states may play a secondary role that has not yet been elucidated. Conversely, teleomorphs are rarely observed in insect pathogens such as *Beauveria bassiana* and *Metarhizium anisopliae* and play an insignificant role in the disease cycle. In some grass endophytes, the sexual state appears to have been lost entirely, perhaps through interspecies hybridization (Scharff et al., 1994), and the infestation is transmitted vertically in the seed of the host grass. Alkaloid toxins produced by the grass endophytes increase the fitness of infested plant hosts by deterring grazers and competitors while promoting plant growth (Bush et al., 1997; Siegel et al., 1987).

Anamorphs play a role in some recently discovered heteroxenous life cycles. Some species of *Harposporium* produce a Hirsutella-like synanamorph (Hodge et al., 1997; Glockling and Dick, 1994b; Glockling and Shimazu, 1997). Some of these species can infect both nematodes and insects, and there appears to be some correlation of spore state with infectivity to the different hosts (Shimazu and Glockling, 1997). Recent studies have revealed even more surprisingly heteroxenous possibilities: Wagner and Lewis (2000) documented the ability of *Beauveria bassiana* to persist as an endophyte in maize, and to attack insects infesting the maize. Species of the genus *Hyperdermium* attack scale insects, then proceed to an endophytic colonization of the host plant (Sullivan et al., 2000). Scale insect pathogens including *Hypocrella schizostachyi*, *H. gaertneriana*, and *H. africana* produce stromata that are disproportionately larger than the original host. Hywel-Jones and Samuels (1998) have suggested that they continued to develop after killing the insect by using the phloem flowing through the dead insect’s mouthparts, thus demonstrating a unique combination of insect and plant parasitism.

3. TAXONOMY

The greatest diversity among anamorphic forms in the Clavicipitaceae is found among the insect pathogenic species. Many of these anamorphs were once considered to be species of *Isaria* or *Spicaria*, two generic names with turbulent nomenclatural histories. The taxonomy and nomenclature of these fungi continue to evolve in light of a modern understanding of biology and phylogeny.
Many telemorphs of the Clavicipitaceae are unispecific genera, and many of these have no known anamorph. *Cordyceps sensu lato*, a group that includes both arthropod-pathogenic and mycoparasitic species, accounts for more anamorphs than all the other telemorphs put together. Its anamorphs fall into more than a dozen form genera, including *Akanthomyces*, *Beauveria*, *Hirsutella*, *Hymenostilbe*, *Metarhizium*, *Nomuraea*, and *Paecilomyces*. Some of its putative anamorphs, including *Polycephalomyces* and *Tilachlidiopsis*, demand further study.

At present, problems in the delimitation of anamorph genera are still being resolved, and the characters of asexual states have had little effect on teleomorph systematics at the generic level. Diehl (1952), however, found anamorphic characters important in subdividing the Clavicipitaceae into subfamilies and tribes. It is to be expected that anamorph morphology can contribute toward our understanding of the taxonomy of sexual forms. Complex anamorphic forms, in particular, appear to have few problems with monophyly; the simplest Verticillium-like forms may have evolved frequently through loss.

### 3.1. Overview of Morphological Features

The dominant modes of conidiogenesis in the Clavicipitaceae are enteroblastic (phialidic) and holoblastic, with sympodial proliferation (Seifert and Gams, 2001). Phialoconidia may be produced in dry chains, or, in other species, in slime balls or cirri. Most species are mononematous, but some form conidiomata including synnemata, coremia, and acervuli. Colors are typically hyaline or bright, but in some species dark structures are formed. Dark conidia are uncommon, but are present in *Desmidiospora*, *Ustilaginoidea*, and in the dark chlamydospores formed by some Verticillium-like species.

Repetitive spore germination is widespread in the family. The ascospores of some *Cordyceps*, *Hypocrella*, *Epichloe*, and *Claviceps* species germinate repetitively (Bacon and Hinton, 1988; Sung, 1996; Hywel-Jones, 1993; Ueker, 1980), each cell forming a minute neck perpendicular to the body of the spore, and bearing one or more small conidia. These states typically lack generic names. Conidia may also germinate repetitively, as they do in the *Ephelis* anamorph of *Balansia claviceps* (Ullasa, 1969); some *Aschersonia* species (Evans, 1994); *Hymenostilbe ventricosa* (Hywel-Jones, 1995a); the *Sphacelia* conidia of *Claviceps paspali* (Luttrell, 1977); *C. cynodontis* (Frederickson et al., 1989); and *C. africana* (Frederickson et al., 1989, 1991). The ecological function of this mode of germination has not been studied; it may improve the likelihood of successful infection of a host.

Resting structures are produced by some species. The ergot sclerotium produced by *Claviceps* species is the best-known example: these dark structures develop in place of an ovary in a grass inflorescence (Stewart, 1957); they germinate to produce a sexual stroma, some first requiring a dormant period.
Some authors consider the mass of mycelium inside a killed host insect to be functionally equivalent to a sclerotium, with the insect’s exoskeleton taking on the role of the rind. Small, dark dictyochlamydospores formed by *Rotiferophthora* and related species and the multicellular aleurioconidia of *Desmidiospora* appear to serve as long-lived diaspores as well. The multicellular, globose diaspores produced by *Hirsutella jonesii* (Speare, 1920a,b) and *H. cryptosclerotium* (Fernández-García, 1990) potentially also serve as survival structures.

### 3.2. Teleomorph Connections

The connection of anamorphic insect pathogens to *Cordyceps* was first made by Tulasne (1857), who wrongly inferred *Paecilomyces farinosus* to be the anamorph of *Cordyceps militaris*. Although corrected by the careful work of de Bary and Petch, this misconception persisted in the literature through the late 1900s.

Many anamorphic taxa remain unconnected with teleomorphs, and cultural studies are badly needed to connect anamorphic and teleomorphic states. The frequency of occurrence of the teleomorph relative to the anamorph varies tremendously among clavicipitaceous fungi. In many *Claviceps* species, the sexual stromata that are produced in spring from over-wintered ergots (sclerotia) are critical initiators of the new infection cycle (Luttrell, 1977). In many insect pathogens, only anamorphic stages are so far known, and it may be that some have lost the ability to form the sexual stage. It is possible that many taxa form the teleomorph only in a certain host, while anamorphic states can infect and produce conidia on a broader range of hosts.

### 4. CULTURE

Culturing of most anamorphs in this group is by routine methods on standard media (Humber, 1997b). In a few cases, special media may be desired for selective isolation or to promote sporulation. In general, the species of *Cordyceps* subg. *Neocordyceps* and *Cordyceps* subg. *Ophiocordyceps* grow slowly and are difficult to isolate. Species of *Cordyceps* subg. *Cordyceps* grow more readily on standard media. The spores of most species germinate well on nutrient media, but some appear to require host or other factors to break dormancy. Some ascospores may be long-lived and slow to germinate: Hodge et al. (1998) observed that ascospores of *C. variabilis* began to germinate only after 1 month on nutrient medium.

Baiting methods have been used successfully for isolation of insect pathogenic fungi, where available, live or killed pupae (the latter the byproducts of silkworm culture) have been used to selectively isolate insect pathogens from soils (Sato et al., 1994). Selective media have been developed for *Metarhizium* and *Beauveria* which appear to be broadly useful for other entomopathogens. These include the copper-based medium of Bååth (1991) and the dodine-based
medium of Beilharz et al. (1982). Their utility has not been tested among the plant-associated species of the Clavicipitaceae. Specific methods for extracting cultures of endophytes have been presented by Bacon (1988).

Connections between anamorphs and their teleomorphs are still lacking in this group. The needed cultural studies were rarely performed by the major workers in the field before the late 1900s. Recent workers have discovered novel connections, but it seems likely that many species, in particular those occurring on very small hosts, may never be connected with a teleomorph.

Anamorph–teleomorph connections are best inferred through careful culturing of ascospores. Germination of the ascospores and their subsequent development should be followed to avoid error caused by mixed infection, contamination, and the occurrence of some clavicipitaceous forms as parasites on their close relatives. Methods of single-spore isolation have been outlined by Samuels (1979) and Luttrell (1979), and general cultural methods by Goettel and Inglis (1997). Ideally, Koch’s postulates (Lacey and Brooks, 1997) should be fulfilled to demonstrate that the isolated fungus is indeed a pathogen or symbiont, but in practice this may prove difficult. Some endophytic species, for example, can be introduced to uninfested hosts only with great difficulty. Determination of appropriate inoculum levels is also a significant problem with pathogenic species: too few infective conidia may fail to gain entry; too many conidia may overwhelm an organism that is not a normal host in nature.

The spontaneous development of teleomorphs in culture is relatively rare, but some insect pathogens have been induced to fruit in artificial culture. Cordyceps militaris (Basith and Madelin, 1968), some species of Torrubiella, and the little-known genus Romanoa (Thirumalachar, 1954) have been observed to form perithecial stromata on semisynthetic media. Sung (1996) induced the formation of perithecial forms in 18 different Cordyceps species from Korea using media composed of sterilized brown rice with added synthetic nutrients, or rice media augmented with sterilized, chopped silkworm pupae. The capacity to produce ascomata may be lost through subculturing (personal observation). Claviceps sclerotia can be induced to produce ascocarps if they are first chilled or rested, then incubated in a moist chamber. Many endophytic fungi can be induced to fruit upon inoculation of the appropriate host, but their typically heterothallic mating system requires fertilization with a strain of the opposite mating type. This may be done by transferring conidia from the opposite mating type using a paint brush (Leuchtmann and Clay, 1989; White et al., 1995; White and Owens, 1992; White and Bultman, 1987).

5. IDENTIFICATION

The identification of clavicipitaceous anamorphs depends primarily on observation of the structures associated with conidium formation. Sections are
not generally needed; wet mounts can be prepared according to standard methods described by Humber (1997a,b), Malloch (1981), and other authors. Endophytes can be visualized inside plant hosts using a simple staining protocol (Clark et al., 1983), but generally spore-forming structures are needed for identification, and these are best examined from host surfaces or artificial culture. General identification guides that cover the full range of fungi discussed here are lacking. Pertinent literature of use in identification is listed under individual genera, below.

6. KEY TO CLAVICIPITACEOUS GENERA

1. Forming synnemata on the host .............................. 2
1'. Not forming synnemata on the host ...................... 18

2. Conidia green in mass ...................................... 21. Metarhizium
2'. Conidia some other color .............................. 3

3. Conidia produced on Aspergillus-like conidiophores that arise from the synnema .......................... 4
3'. Not as above .................................. 5

4. Conidiogenous cells phialidic; on spiders ............ 12. Gibellula
4'. Conidiogenous cells polyblastic, producing ameroconidia singly on short denticles. ..................... 30. Pseudogibellula

5. Conidiogenous cells occurring in a compact hymenial layer on well-formed, discrete synnemata .......................... 6
5'. Synnemata loosely arranged, often with a fluffy appearance .......................... 10

6. Conidiogenous cells phialidic ................................ 7
6'. Conidiogenous cells polyblastic .......................... 9

7. Conidia in dry chains. Conidiogenous cells with very short or absent necks, sometimes ornamented .... 1. Akanthomyces
7'. Conidia held in slime, conidiogenous cells with distinct necks .......................... 8

8. Conidiogenous cells with inflated base and a pronounced, usually elongate neck; conidia often held in a discrete slime droplet .............................. 17. Hirsutella
8'. Conidiogenous cells tapering, conidia produced in slime that may coalesce with that of adjacent conidiogenous cells ........................................ 29. Polycephalomyces

9. Conidiogenous cells irregularly cylindrical or convoluted; on spiders, usually co-occurring with Gibellula synanamorph .......................... 13. Granulomanus
9'. Conidiogenous cells cylindrical to clavate, bearing multiple apical or subapical denticles; well organized in a hymenial layer on synnema ........................................ 18. Hymenostilbe

10. Conidiogenous cells phialidic ................................ 11
10'. Conidiogenous cells polyblastic? ...................... 16
11. Conidia produced in dry chains ........................................... 12
11'. Conidia produced in slime ............................................... 15
12. Conidiogenous cells tapering into a short apical neck ............... 13
12'. Conidiogenous cells with a blunt or slightly clavate apex ...... 14
13. Conidiophores with a central axis bearing multiple levels of
    whorls of short branches, each bearing 3–7 conidiogenous
    cells ................................................................. 24. Nomuraea
13'. Conidiophores branching; conidiogenous cells short
    flask-shaped. ..................................................... 25. Paecilomyces
14. Conidiogenous cells cylindric with a broad apex, digitate, producing
    chains of conidia appressed along their length that give a prismatic
    appearance to mature specimens and cultures . . . . 21. Metarhizium
14'. Conidiogenous cells subglobose to flask-shaped with short
    necks ............................................................... 1. Akanthomyces
15. Conidiogenous cells long and aculeate, produced in clusters in the
    capitulum of the synnema and laterally . . . . 29. Polycephalomyces
15'. Conidiogenous cells basally cylindrical and narrowing abruptly near
    the apex into a short neck ........................................ 36. Syngliocladium
16. Conidiogenous cells sympodially proliferating; inflated at the base
    and terminating in a minute zigzag rachis . . . . 5. Beauveria
16'. Conidiogenous cells with multiple short denticles ................. 17
17. Conidiophores branching in a penicillate or Paecilomyces-like
    fashion .......................................................... 26. Paraisaria
17'. Conidiogenous cells produced directly from surface of synnema
    or host, lacking macronematous conidiophores; denticles
    minute. .............................................................. 13. Granulomanus
18. Conidia formed on or in a sclerotium or hard, dark macroscopic
    resting structure. .................................................. 19
18'. No sclerotium present .................................................... 20
19. Conidia hyaline or white to orange in mass, ovoid to cylindrical,
    formed sparsely on mononematous conidiophores on an ergot
    sclerotium; on grasses ........................................... 35. Sphacelia
19'. Conidia dark green or brown, subglobose, verruculose, resembling
    the teliospores of a smut fungus; on grasses . . . 39. Ustilaginoidea
20. Conidiomata pycnidia or acervuli .................................... 21
20'. Conidia arising from mononematous conidiophores ............... 24
21. Conidia scolecosporous, three-celled, with terminal cells
    slightly inflated. Causing a witches’-broom disease of
    bamboos ............................................................ 2. Albomyces
21'. Conidia one-celled (in Aschersonia, oil drops may cause the conidia
    to appear multicellular) .......................................... 22
22. Conidiomata pycnidial, conidia fusoid, often brightly colored in mass, produced in copious slime, arising from a stroma enveloping a scale insect or whitefly. .......................... 4. Aschersonia
22'. Conidiomata acervular, on grasses .................. 23
23'. Conidia produced in copious sugary slime on the inflorescences of grasses or on ergot sclerotia .................. 35. Sphacelia
24. Resting structures formed: relatively thick-walled, hyaline or dark spores or macroscopic structures with one or more cells ...... 25
24'. No resting structures present .......................... 33
25. Resting spores microscopic, few-celled .................. 26
25'. Resting structures macroscopic, resembling sclerotia or bulbils .............................................. 31
26. Resting spores one-celled, variously colored ............ 27
26'. Resting spores multicellular, usually darkly pigmented ...... 29
27. Resting spores formed as chains of cylindrical arthroconidia ........................................... 16. Harposporium
27'. No arthroconidia present .................................. 28
28. Resting spores smooth, subglobose, hyaline to orange, formed in dense masses inside an insect host ................. 34. Sorosporella
28'. Resting spores verruculose, subglobose, dark, formed in and on a sclerotium-like body replacing a grass ovary . . . 39. Ustilaginoidea
29. Resting spores very large aleurioconidia with a lobed, dichotomous structure. Formed singly on slender stalks on the body of a dead ant host. ........................................ 7. Desmidiospora
29'. Resting spores not as above .............................. 30
30. Resting spores flattened ................................. 32. Rotiferophthora
30'. Resting spores three-dimensional dictyochlamydospores ...... 31
31'. Conidia of phialidic synanamorph bearing an adhesive hapteron ........................................... 14. Haptocillium
31. Conidia of phialidic synanamorph lacking a hapteron ............................................. 28. Pochonia
32. Resting structures black, produced in inflorescences of a grass host ............................................ 33
32'. Resting structures subglobose bulbils composed of densely packed hyphae, on the cadaver of insect hosts ........ 17. Hirsutella
33. Resting structure a true sclerotium (ergot) with a light-colored interior and a black rind, a phialidic anamorph sometimes present at the apex .................................. 35. Sphacelia
33'. Resting structure loosely constructed and sclerotium-like. Dark, subglobose, verruculose spores often found intermingled and on the surface .............................................. 39. Ustilaginoidea

34. Conidia helically coiled .............................................. 35

34'. Conidia not helically coiled .............................................. 36

35. Conidia produced in small numbers from conidiogenous cells with a subglobose base and narrow neck. On nematodes, rotifers, or tardigrades. .............................................. 16. Harposporium

35'. Conidia produced singly, laterally on the stipe of a synnema on an insect body ................................. 17. Hirsutella petchabunensis

36. Conidiogenous cells polyblastic; producing conidia from more than one conidiogenous locus .............................................. 56

36'. Conidiogenous cells with a single conidiogenous locus, most often an enteroblastic phialide .............................................. 37

37. Conidia produced in dry chains .............................................. 38

37'. Conidia not produced in chains, or produced in slime .............................................. 44

38. No complex conidiophores present; conidiogenous cells simple aculeate phialides that occur singly or in whorls .............................................. 42

38'. Conidiophores more complex .............................................. 39

39. Conidia produced from a palisade-like layer of conidiogenous cells; conidial chains accumulating to form a prismatic mass, usually in shades of green .............................................. 21. Metarhizium

39'. Conidiophores not forming a hymenial layer, conidia green or other colors .............................................. 40

40. Conidiophores penicillate or brush-like, conidiogenous cells flask-shaped, conidia in divergent chains, usually in shades of white to lilac; on insects .............................................. 25. Paecilomyces sect. Isarioidea

40'. Conidiophores not as above .............................................. 41

41. Conidiophores bearing at several levels whorls of very short branches, each bearing a whorl of flask-shaped conidiogenous cells with slender necks. In shades of green or lilac. On insects or spiders .............................................. 24. Nomuraea

41'. Conidiophores terminating in a swollen vesicle on which many short, flask-shaped conidiogenous cells are arrayed. In nests of leaf-cutting ants .............................................. 11. Escovopsis

42. Conidia with a mucous hapteron, which may appear as an apical wall thickening. Conidia in short chains or slimy drops. Parasites of rotifers .............................................. 14. Haptocillium

42'. Conidia lacking a hapteron .............................................. 43

43. Conidiogenous cells in whorls of two or more, sometimes single .............................................. 20. Lecanicillium
43'. Conidiogenous cells single, at right angle to subtending hypha ........................................ 33. Simplicillium
44. Conidiogenous cells short flask-shaped, narrowing to a slender neck ........................................ 47
44'. Conidiogenous cells other shapes ........................................ 45
45. Conidiogenous cells cylindrical or aculeate, not thickened at the base ........................................ 52
45'. Conidiogenous cells other shapes ........................................ 46
46. Conidiogenous cells integrated in a linear conidiophore, each producing clavate conidia from a single short neck adjacent to a septum ........................ ........ 8. Drechmeria
46'. Conidiogenous cells inside the body of a nematode host. Only the short, cylindrical conidiogenous necks protrude through the host cuticle ........................................ 27. Plesiospora
47. Conidiogenous cells with a conspicuous collarette, conidia with a thin, filamentous appendage .......................... 15. Haptospora
47'. Conidiogenous cells with subglobose base and a narrow, cylindrical neck, without a collarette ........................................ 48
48. Conidiogenous cells produced singly on the mycelium, with cylindric to ellipsoid base tapering abruptly to a pronounced, slender neck. Conidia borne singly or in small groups in a drop of persistent slime. Slime sometimes appears as a roughened texture on the conidia. On insects, mites and nematodes ........ 17. Hirsutella
48'. Conidiogenous cells produced on differentiated conidiophores ........................................ 49
49. Conidiogenous cells produced laterally and terminally on a simple conidiophore from the body of a nematode, rotifer or tardigrade; conidia often helical, crescent-, jack-, or shoe-shaped ........................................ 16. Harposporium
49'. Not as above ........................................ 50
50. Conidiophore tree-shaped, resembling that of a Trichoderma or Beauveria, bearing terminal and lateral conidiogenous cells with a subglobose base and short, narrow, hooked neck. Colonies usually white. From soil or insects ........................................ 38. Tolypocladium
50'. Conidiophore less complex ........................................ 51
51. Conidiophore bearing scattered terminal and lateral conidiogenous cells with a cylindrical to ellipsoid base and short neck. From aquatic flies or rotifers ........................................ 6. Culicinomyces
51'. Conidiophore bearing whorls or pairs of conidiogenous cells with a cylindrical base. Conidia cylindric and produced in copious slime. Synnemata often present. On insects ........ 36. Syngliocladium
52. Species occurring on grass plants, inflorescences, or seeds. Rarely isolated from soil ........................................ 53

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52'. Species occurring on arthropod, nematode, or other animal hosts, sometimes isolated from soil
53. Conidiophores penicillate or verticillate. Occurring on the inflorescences of grasses or on ergot sclerotia. Conidia produced in copious slime
53'. Conidiophores micronematous, conidiogenous cells simple, aculeate, arising at right angles to the subtending hyphae. Occurring in grass tissues or on the surface of infested plants 23. *Neotyphodium*
54. Conidia with a small adhesive hapteron which is sometimes apparent as an apical thickening of the conidial wall . . . . . . 14. *Haptocillium*
54'. Conidia lacking hapteron ................................................. 55
55. Conidiogenous cells in whorls of two or more, sometimes single ........................................... 20. *Lecanicillium*
55'. Conidiogenous cells single, at right angle to subtending hypha ............................................. 33. *Simplicillium*
56. Conidiogenous cells with an ellipsoid or subglobose base and a slender neck that is prolonged into a zigzag rachis ........57
56'. Rachis absent ................................................................. 58
57. Conidiogenous cells with subglobose base. On insects .................................................. *Beauveria* and *Microhilum*
57'. Narrow rachis emerges laterally or terminally from conidiogenous cells integrated in the conidiophore. On rotifers . . . 31. *Pseudomeria*
58. Conidia arising from inconspicuous denticles on the upper portion of a cylindrical conidiogenous cell. On spiders . . . 13. *Granulomanus*
58'. Conidia arising from multiple necks on an elongated conidiogenous cell ............................................. 17. *Hirsutella*

7. THE GENERA

*Type species*: *Akanthomyces aculeata* Lebert.

*Known teleomorphs*: *Cordyceps* and *Torrubiella* species.

*Diagnosis*: Colonies slow-growing, typically white to cream, becoming setose with synnemata. Conidiomata synnematous; terete, usually white to cream, sometimes darkened toward the base, bearing a hymenium-like palisade of phialidic conidiogenous cells over their entire surface, or over a fertile region surmounting a short stipe. Mononematous conidiogenous cells sometimes produced sparsely in culture, and when present, longer and narrower than those found on conidiomata. Synnematous conidiogenous cells subglobose to ellipsoid to conical with a short, narrow neck or without a neck, sometimes verruculose.
Conidia single-celled, hyaline, shape variable among species (ellipsoid to clavate to cylindrical), produced in dry chains.

About 10 species are known, which include pathogens of Lepidoptera, Coleoptera, and Araneida (spiders). A. johnsonii was reported as a saprobe or fungicolous species in leaf litter, but arthropod parasitism is unknown (Vincent et al., 1988). None of the species is well characterized in terms of its ecology, and none has been assessed as a biocontrol agent.

Early concepts of Akanthomyces were somewhat confused, especially with respect to its relationship with Hymenostilbe (Petch, 1933). Mains (1950b), Samson and Evans (1974), and Wywel-Jones (1996b) clarified its circumscription. The genus Insecticola Mains was segregated from Akanthomyces by Mains (1950b) based on the sterile stalk of the synnema, and the different origins of the conidiogenous cells. Samson and Evans (1974) felt that the continuum of variation in these characters did not support the distinction. Three species were included in Insecticola by Mains (1950b) (I. clavata, I. fragilis, and I. pistillariaeformis); a single additional species (I. peruamazonensis Matsushima) was added by Matsushima (1993). Of these, only I. pistillariaeformis has a valid name as an Akanthomyces; the remaining three are transferred to Akanthomyces as follows: Akanthomyces clavata (Mains) K. T. Hodge comb. nov. (Basionym, Insecticola clavata Mains, Mycologia 42:577, 1950); Akanthomyces fragilis (Petch) K. T. Hodge comb. nov. [Basionym, Hymenostilbe fragilis Petch, Trans Br Mycol Soc 21:56, 1937; Insecticola fragilis (Petch) Mains]; Akanthomyces peruamazonensis (Matsush.) K. T. Hodge comb. nov. (Basionym, Insecticola peruamazonensis Matsush., Matsushima Mycol Mem. 7:55, 1993).

A. pistillariaeformis (Pat.) Samson & Evans is the most frequently collected species of Akanthomyces. It occurs on large adult sphingid moths throughout the tropics and subtropics and is the anamorph of Cordyceps tuberculata. The spider pathogen A. araneearum appears to be the anamorph of C. thaxteri (Mains, 1950a,b), and another spider pathogen, A. arachnophilus (Petch) Samson & Evans, appears to be the anamorph of Torrubiella flava Petch (Samson and Evans, 1974; Petch, 1923). Teleomorph connections in this genus are largely anecdotal and have yet to be confirmed through cultural study.


Type species: Albomyces take Miyake.
Known teleomorph: Aciculosporium.
Diagnosis: Conidiomata formed in a pseudoparenchymatous stroma enclosed by and incorporating living bamboo leaf sheaths and twigs. Irregular conidiomatal locules form on the inner surfaces of bamboo leaf
sheaths. Filiform conidiogenous cells line the irregular cavities, and are holoblastic and sympodial as in Ephelis. Conidia filiform, hyaline, typically three-celled, with a long-cylindrical central cell and slightly inflated, refractive terminal cells. The conidia are produced in a matrix of hydrophilic slime that is clear to milky and dries white to orange; they are extruded through lateral openings and at the apex of the stroma. The conidia germinate at each end on agar media to produce curved, slender, dichotomously branching appendages. Growth in culture yeasty, comprising mostly secondary conidiation; of limited extent.

Only two Asian species are known, A. take Miyake (anamorph of Aciculosporium take I. Miyake) and A. sasicola Oguchi [anamorph of Aciculosporium sasicola Oguchi (2001)]. These fungi cause a economically important witches'-broom disease of bamboos in eastern Asia. The natural host range of A. take has been studied by Tsuda et al. (1997) and includes at least 17 bamboo species. The infectivity of the conidia to bamboo was demonstrated by Kao and Leu (1976). The curious dichotomous conidial appendages that arise upon germination may aid in splash dispersal (Tsuda et al., 1997). A similar pattern of germination was recorded for the Ephelis-like anamorph of Neoclaviceps (Sullivan et al., 2001).

Albomyces and Ephelis are closely allied; Albomyces is distinct in its three-celled conidia and the enclosed nature of the conidiogenous surface. The generic name Albomyces was invalidly published by Hino (1962), who failed to specify a type species as required by Article 37.1. This problem should be repaired if Albomyces is found to be distinct from Ephelis.

7.3. Aphanocladium W. Gams, Cephalosporium-artige Schimmelpilze (Hyphomycetes), p 196, 1971

Type species: Aphanocladium album (Preuss) W. Gams.
Known teleomorphs: None.

The genus can be recognized by its production of conidiogenous cells that are either narrowly flask-shaped or reduced to intercalary terete denticles that collapse and become empty of cytoplasm soon after producing a solitary aeroconidium (Gams et al., 1984, 1998). However, the relationships of Aphanocladium species are not well resolved, and molecular approaches have revealed the genus to be polyphyletic (Gams et al., 1998; Zare et al., 2000; Sung et al., 2001). Some species, including the type species A. album, have been removed to genera allied to other families of the Hypocreales sensu stricto (Gams et al., 1998). Thus only A. araneearum and A. macrosorum are possible members of the Clavicipitaceae (data are lacking in the second case). If so, they need to be removed from Aphanocladium following thorough molecular and morphological study.

*Type species:* *Aschersonia taitensis* Mont.

*Known teleomorphs:* *Hypocrella*.

*Diagnosis:* Conidiomata formed in stromata arising from the body of a scale insect (Coccoidea) or whitefly (Aleyrodidae), which is often completely obscured by the fungus. Stromata variable, but frequently pulvinate, variously colored, bearing pycnial or acervular conidiomata. Filiform conidiogenous cells that sometimes branch at acute angles line the conidiomata. Conidia small, fusiform, one-celled although sometimes containing oil droplets that give the illusion of multicellularity, produced enteroblastically and oozing from the conidiomata in copious slime, often brightly colored in mass. In some species, long, slender, filiform paraphyses arise among the conidiogenous cells and may protrude from the conidiomata.

*Aschersonia* species are anamorphs of *Hypocrella* species, all of which are parasites of scale insects and whiteflies (Evans and Hywel-Jones, 1997; Petch, 1921). *Aschersonia* is clearly defined by its coelomycetous habit. The gross morphology of the conidiomata is quite variable, ranging from uniformly flask-shaped to irregularly convoluted. Cirri of slimy, often brightly colored conidia are frequently present on the surface of the stroma. Key factors that differentiate species include spore size, color of the spore mass, and color and texture of the stroma. Petch (1921) erected two subgenera. He felt that *Aschersonia* subg. *Aschersonia* Petch occurred on aleyrodids and had paraphysate conidiomata, and *Aschersonia* subg. *Leprieuria* Petch occurred on scale insects and lacked paraphyses. Some exceptions to Petch’s classification have been found (Dingley, 1954; Tzean et al., 1997). A synanamorph has been occasionally observed, but has not been well characterized. Petch (1921) and Parkin (1906) observed in *Hypocrella reineckiana* a “bloom” on young stromata due to the production of scattered conidiophores bearing small conidia. The conidia (Evans, 1994), and ascospores of the *Hypocrella* teleomorphs (Hywel-Jones and Evans, 1993) sometimes germinate repetitively.

*Aschersonia* species were among the first fungi used as biological controls in North America, where *A. aleyrodis* was used to control citrus scale in Florida (Fawcett, 1908, 1936). The use of other species as biocontrols has scarcely been investigated. No modern taxonomic treatment of the genus exists. Petch treated about two dozen species in 1921; Mains reviewed North American species (Mains, 1959a,b). The closest allies of *Aschersonia* morphologically are species of *Ephelis*, which, however, produce conidia superficially on pulvinate or apothecioid stromata and are symbionts of grasses.

*Type species:* *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin.  
*Known teleomorphs:* *Cordyceps* species.  
*Diagnosis:* Cultures typically fast-growing, white, and becoming powdery from an accumulation of conidia, which often adhere in small spheres. Some strains produce a red pigment (oosporein, see Vining et al., 1962) in the medium. Conidiophores simple or with a simple whorled branching structure, producing dense whorls of conidiogenous cells. Conidiogenous cells have subglobose to cylindrical bases that taper abruptly into a narrow neck which develops into a slender rachis through sympodial development. Conidia single-celled, small, subglobose to cylindrical, hyaline, dry.

*Beauveria* is well distinguished by its basally inflated conidiogenous cells from which conidia are sympodially produced on a slender rachis that increases in length with age. It closely resembles the unspecific genus *Microhilum.* The spores are typically small and white, and produced from clustered conidiogenous cells that may aggregate in visible spore balls. *Cordyceps* teleomorphs have recently been discovered for *B. bassiana* (Li et al., 2001), *B. brongniartii* (Shimazu et al., 1988), and *B. sobolifera* (Liu et al., 2001).

About eight species are known. *Beauveria bassiana* is certainly the most important species in terms of insect biocontrol applications (Li and Yan, 1992; Diehl-Fleig et al., 1993; Ferron, 1978; Rombach et al., 1986a). It exhibits a worldwide distribution and attacks an extremely broad range of host insects, suggesting the possibility of cryptic species. *B. bassiana* may opportunistically infect vertebrates, including humans (Kisla et al., 2000) and even alligators (Fromtling et al., 1979). Individual isolates often exhibit a narrower range of host specificity than is known for the species overall. *B. brongniartii* is a pathogen of scarabaeid beetle larvae, and has also been used successfully for biocontrol.


*Type species:* *Culicinomyces clavisporus* Couch, Romney & B. Rao.  
*Known teleomorphs:* None.  
*Diagnosis:* Cultures slow-growing. Conidiophores simple or with a simple whorled branching structure. Conidiogenous cells phialides with ellipsoid bases that taper abruptly into a narrow neck. Conidia single-celled, subglobose to cylindrical, hyaline, produced in slimy heads.

Two or three species are accepted: *Culicinomyces bisporalis* and *C. clavisporus* are pathogens of flies (Diptera) in aquatic habitats (Sigler et al.,
1987; Goettel et al., 1984; Couch et al., 1974); C. parasiticus parasitizes rotifers (Bissett, 1983). The latter species was transferred from Tolypocladium by Sigler et al. (1987), and indeed the two genera are very similar. C. clavisporus has been considered as a potential biocontrol of mosquito larvae in aquatic habitats (Sweeney et al., 1973).

7.7. Desmidiospora Thaxter, Bot. Gaz. 16:203, 1891

Type species: Desmidiospora myrmecophila Thaxter.
Known teleomorphs: Cordyceps unilateralis.

Diagnosis: Cultural characteristics unknown. Conidia aleuriospores, dark brown, thick-walled, multicellular, dichotomously lobed, large (80–100 μm), arising from unpigmented hyphal stalks on the surface of the insect host.

This unspecific genus is characterized by its flat, brown, dichotomously lobed dictyoconidia, and is an occasional synanamorph of the ant pathogen Hirsutella formicarum (Thaxter, 1891; Evans and Samson, 1984). It is so far known from very few collections in America and Africa, and is limited to carpenter ants in the genus Camponotus (Thaxter, 1891; Evans and Samson, 1984; Clark and Prusso, 1986). Evans and Samson (1984) speculated that it might be preferentially formed on queens, and that it is adapted for survival in the secluded habitats in which infected queens are usually found (in contrast to the exposed locations of workers killed by H. formicarum). When he first described it, Thaxter (1891) suggested that it might be an anamorph of C. unilateralis, specimens of which had been collected nearby. This fact has been borne out by modern collections.


Type species: Drechmeria coniospora (Drechsler) W. Gams & Jansson.
Known teleomorphs: None.

Diagnosis: Colonies very slow-growing, white. Conidiophores erect, simple, the terminal cell tapering to a short conidiogenous neck. Subtending cells each bear a short conidiogenous neck below the upper septum. Several conidia are produced from each neck; they accumulate in small groups near the neck opening. Conidia one-celled, hyaline, smooth, conical or falcate, with a sticky hapteron that facilitates attachment to the host.

Two species of Drechmeria are known: D. coniospora is a common and cosmopolitan pathogen of nematodes; the protozoan pathogen D. harposporioides
(Gams and Jansson, 1985; Barron and Szijarto, 1982) has rarely been observed. No teleomorphs are known in this group, but a link to the Clavicipitaceae has been established through molecular comparisons (Gernandt and Stone, 1999). The genus is further discussed in this volume by Gams and Zare (Chapter 2).


*Type species:* *Engyodontium parvisporum* (T. Petch) G. S. de Hoog.

*Known teleomorphs:* None.

*Engyodontium* is close to *Aphanocladium, Acremonium,* and *Lecanicillium.* Two types of conidiogenous cells are produced: Slender, aculeate phialides resemble those of *Acremonium,* and produce conidia in chains or in small slime balls. Conidia are also produced on short necks or denticles that arise directly from undifferentiated mycelium (Gams et al., 1998). Gams and Zare (2001) noted that species currently included in *Engyodontium* differ in the structure of their conidiogenous cells and are probably not congeneric, so the current disposition of most *Engyodontium* species is uncertain. *E. aranearum* is now considered a synonym of *Lecanicillium tenuipes,* and *E. arachnophilum* is now called *L. aranearum* (Zare and Gams, 2001a). The type species *E. parvisporum* and others were considered likely relatives of *Beauveria* by Zare and Gams (2001a).

7.10. **Ephelis E. M. Fries, Summa Veg. Scand. 370, 1849**

*Type species:* *Ephelis mexicana* E. M. Fries.

*Known teleomorphs:* *Epichloe, Atkinsonella, Balansia, Myriogenospora,* *Neoclaviceps.*

*Diagnosis:* Conidiomata sporodochial, flat or concave, on the surface of a grass host. Conidiogenous cells arising as a palisade layer; slender, filiform. Conidia produced holoblastically. Multiple conidia arise from a conidiogenous cell through sympodial proliferation, although the internodes of the sympodulae are frequently very short and may be difficult to observe, so the conidia appear to arise in a whorl from a single locus (Rykard et al., 1984).

*Ephelis* species resemble *Aschersonia* species in forming discrete conidiomata; the latter are insect pathogens and form conidia in slime masses or cirri. In some *Balansia* species, Diehl (1950) reported a phialidic synanamorph perhaps referable to *Neotyphodium.* Later authors have questioned his findings (Rykard et al., 1984), although *Neotyphodium* and *Ephelis* synanamorphs do occur in *Atkinsonella* (Morgan-Jones and White, 1989).
Ephelis conidia can serve as gametes in *Atkinsonella texensis* (Leuchtmann and Clay, 1989) and *Balansia epichloe* (White, 1993). The conidia have been observed to germinate repetitively in the Ephelis anamorphs of *Balansia claviceps* (Ullasa, 1969), *Balansia aristidae* (Phelps and Morgan-Jones, 1993) and others (Diehl, 1950). Ephelis-like conidia that proliferate through repetitive germination occur in cultures of *Neoclaviceps* (Sullivan et al., 2001) and *Aciculosporium* (anamorph *Albomyces*) (Kao and Leu, 1976).

Kuldau et al. (1997) used molecular data to demonstrate that a sample of fungi with Ephelis anamorphs formed a monophyletic group within the Clavicipitaceae subfamily Clavicipitoideae Diehl, confirming Diehl’s (1950) use of anamorphic characters to delineate tribes of the family Clavicipitaceae. Many Ephelis states have not been named, and few identification resources focus on Ephelis states (but see Govindu and Thirumalachar, 1961).

**7.11. Escovopsis Muchojev & Della Lucia, Mycotaxon 37:192, 1990**

*Type species:* *Escovopsis weberi* Muchovej & Della Lucia.

*Known teleomorphs:* None.

*Diagnosis:* Colonies fast-growing, yellow to olivaceous brown. Sporulation mononematous. Conidiophores broad-celled, branching at right angles, each branch terminating in an *Aspergillus*-like globose or clavate vesicle bearing phialides. Conidiogenous cells short flask-shaped, tapering into a short neck, and producing chains of dry conidia. Conidia single-celled, hyaline to lightly colored.

The species of this unusual genus are parasites (“weeds”) in the fungus gardens of leaf-cutting ants (Formicidae: Attini) in Central and South America. These ants cultivate lepiotaceous fungi in underground gardens (Weber, 1966). The precise nature of the interaction of Escovopsis species with the cultivated fungi is unknown, but when uncontrolled, an Escovopsis species will overrun the other fungus and cause abandonment of the garden by the ants (Currie et al., 1999a,b). Presumably they act as mycoparasites or competitors. They are controlled by the ants using a fungicidal toxin derived from a commensal actinomycete that grows on the ventral surface of the workers. This remarkable symbiosis reflects a long co-evolutionary history (Currie et al., 1999a,b; Currie, 2001), and a unique life history among clavicipitaceous fungi.

Some features of Escovopsis species resemble those of *Gibellula* and *Tolypocladium*, but the genus is unique in terms of its gross morphology, fast growth in culture, and ecological niche. Its relationship to the Clavicipitaceae is suggested by molecular data; more studies are needed. Two species, *E. weberi* (Muchovej and Della Lucia, 1990) and *E. aspergillioides* (Seifert et al., 1995) are
currently known; Currie (2001) suggests that others remain to be described from similar habitats.


*Type species:* *Gibellula pulchra* (P. A. Saccardo) Cavara.

*Known teleomorphs:* *Torrubiella* species.

*Diagnosis:* Colonies very slow growing on standard media, white to pinkish tan. Sporulation typically synnematous but sometimes mononematous. Conidiophores typically arising at right angles from the surface of the synnema, *Aspergillus*-like, consisting of a simple verruculose stipe bearing an inflated apical vesicle from which a series of cylindrical or inflated metulae arise, each metula bearing one or a few cylindrical to flask-shaped conidiogenous cells. Conidiogenous cells flask-shaped phialides which taper abruptly at the apex and produce conidia in dry chains. Conidia single-celled, hyaline, smooth-walled, and ellipsoid to fusiform. A *Granulomanus* synanamorph is frequently present.

The species of this distinctive genus are pathogens of spiders (Evans and Samson, 1987). They are quickly recognized by their conidiophores, which superficially resemble those of *Aspergillus* and are most often borne on synnemata. Most species have proved difficult to grow in pure culture. A *Granulomanus* synanamorph frequently appears on the host body and occasionally on the synnemata.

About 16 species are currently accepted, but additional tropical species are expected. Two common species in temperate climates worldwide differ chiefly in the length of the conidiophores: *G. leiopus* and *G. pulchra* (Mains, 1950a). One interesting tropical species, *G. alata* (Samson et al., 1988) which is perhaps involved in wind dispersal. Several species are known to have *Torrubiella* teleomorphs.


*Type species:* *Granulomanus aranearum* (Petch) de Hoog & Samson.

*Known teleomorphs:* *Torrubiella*.

*Diagnosis:* Cultural characters unknown. Sporulation typically mononematous but sometimes synnematous. Conidiogenous cells typically arising from the host surface, cylindrical, polyblastic, developing many short necks or denticles, each of which produces a single, dry conidium. These conidiogenous cells sometimes arise on *Aspergillus*-like heads
typical of the *Gibellula* synanamorph. Conidia single-celled, hyaline, smooth-walled, and narrow ellipsoid to fusoid. A *Gibellula* synanamorph is usually present.

*Granulomanus* states typically co-occur with their *Gibellula* synanamorphs on spider hosts and many have not been named separately. The conidiogenous cells usually cover the surface of the host body, but sometimes appear on synnemata. Humber and Rombach (1987) considered *Granulomanus* a synonym of *Gibellula* based on frequent co-occurrence, but it seems more useful to treat the two genera separately. *Granulomanus* conidia are presumed to be infective, but their role in the disease cycle has not been demonstrated experimentally, and it is possible that they function as spermatia.


*Type species*: *Haptocillium balanoides* (Drechsler) Zare & W. Gams.

*Teleomorph*: None known.

*Diagnosis*: Colonies slow-growing. Conidiophores erect or prostrate, bearing whorled or solitary phialides with an inflated base. Conidia variously shaped, with an adhesive hapteron, produced in slimy heads or short chains or both. Dictyochlamydospores sometimes present.

The type species, *H. balanoides*, was until recently classified as *Verticillium balanoides*. Recent work confirmed the need to segregate it and other *Haptocillium* species from the former genus (Gams and Zare, 2001; Zare and Gams, 2001b). Most species attack nematodes, infecting after adhesive spores attach to the cuticle. The genus is discussed further in this volume by Gams and Zare (Chapter 2).


*Type species*: *Haptospora appendiculata* Barron.

*Known teleomorphs*: None.

*Diagnosis*: Characteristics in axenic culture unknown. Conidiophores simple or sparsely branched bearing single or groups of conidiogenous cells. Conidiogenous cells phialides, flask-shaped, with a membranous collarette, intercalary phialides occasionally present. Conidia one-celled, hyaline, with a filamentous basal appendage.

This genus is known from a single species that attacks rotifers (Digononta: Bdelloidea). It resembles *Phialophora* in forming distinct, membranous collarettes, and *Harposporium* in the arrangement of its conidiophores and

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parasitic habit. The link to the Clavicipitaceae has not been confirmed through
cultural or molecular studies, but is assumed from similarities in its morphology
and life cycle. Haptospora is treated further in this volume by Gams and Zare
(Chapter 2).


*Type species*: Harposporium anguillulae Lohde.

*Known teleomorphs*: Atricordyceps.

*Diagnosis*: Colonies slow-growing, white to tan to olivaceous brown.
Sporulation mononematous. Conidiophores simple or sparsely branched,
bearing flask-shaped phialides which taper into a short neck. Conidia
typically single-celled, hyaline, smooth-walled, and of unusual shape:
often sickle-shaped, helically coiled, or shaped like a women’s pump-
style shoe or jack. A small adhesive hapteron may be produced on the apex
of the conidium. A small number of conidia accumulate on each phialide.

The conidiogenous cells of this genus closely resemble those of
*Toxypocladium*. Harposporium species, however, generally grow more slowly
and are darker in culture. They are readily recognized by the unusual shapes of
their conidia, which apparently facilitate infection when caught in the digestive
tracts of their nematode, tardigrade, and rotifer hosts.

Harposporium species are well known as nematode and rotifer parasites,
thanks to the work of Drechsler, Barron, and others. They are covered in greater
detail herein in Chapter 2. Two species are known to exhibit heteroxenous life
histories: The only known Harposporium teleomorph, Atricordyceps harposporoi
des Samuels (Samuels, 1983), was collected on an arthropod inferred to be a
millipede; *H. janus* is capable of parasitizing both insects and nematodes
(Shimazu and Glockling, 1997), and produces a Hirsutella-like synanamorph that
may be involved in attacking the insect host. Other Harposporium species are
known to produce similar synanamorphs (Hodge, 1997); their host ranges should
be examined more closely.

7.17. **Hirsutella Pat., Rev. Mycol. (Toulouse) 14:67, 1892**

*Type species*: Hirsutella entomophila Pat.

*Known teleomorphs*: Cordyceps, Cordycepioideus, Torrubiella.

*Diagnosis*: Colonies slow- to fast-growing, white to tan to dark brown.
Sporulation synnematous in some species with known or suspected
Cordyceps anamorphs, strictly mononematous in others. Conidiogenous
cells phialides, cylindrical to flask-shaped, tapering abruptly into one or several slender necks, on which one or a few conidia accumulate in a persistent subglobose slime drop. In some species the slime drop darkens with age; in others it is so scanty that it appears as a roughening of the conidial wall. Conidia one- or two-celled, mostly either bluntly fusiform or subglobose, hyaline, smooth-walled, or appearing rough due to irregular distribution of the slime. Insecticolous.

_Hirsutella_ was erected by Patouillard (1892) based on a synnematous species. Many mononematous species have since been included (Minter and Brady, 1980), although Mains (1951) questioned their inclusion in _Hirsutella_. The genus now includes over 80 species with diverse morphological attributes. It is likely that these will be segregated into different genera in the future. The current concept of _Hirsutella_ intergrades with that of _Hymenostilbe_ in cases such as the anamorph of _C. clavulata_ (Mains, 1958), in which the conidiogenous cells are _Hirsutella_-like when young, but develop more conidiogenous loci as they mature. A similar condition seen in the _Hirsutella_-like synanamorphs has been recorded for some _Harposporium_ species (Hodge et al., 1997; Glockling and Shimazu, 1997). Further taxonomic work is needed. Currently no identification resources cover all species of the genus.

_Hirsutella thompsonii_ (McCoy, 1981) and _H. rhossiliensis_ (Jaffee, 1992) have been used as biocontrols of mite and nematode pests, respectively. Other species have also been proposed as biocontrol agents (see, e.g., Li and Yan, 1992; Evans et al., 1999; Greenwood and Mills, 1989; Ochiel et al., 1997), but few have been developed on a commercial scale.

Most known _Hirsutella_ teleomorphs are species of _Cordyceps_ or _Torrubiella_. Teleomorph connections include _C. aphodii_ (Mathieson, 1949), _C. atewensis_ (Samson et al., 1982), _C. brunneapunctata_ (Hywel-Jones, 1995b), _C. clavulata_ (Mains, 1958), _C. khaoyaiensis_ (Hywel-Jones, 1994), _C. pseudomilitaris_ (Hywel-Jones, 1994), _C. rubripunctata_ (Samson et al., 1982), _C. stylophora_ (Mains, 1941), _Torrubiella hirsutellae_ (Petch, 1937), _T. iriomoteana_ (Hywel-Jones, 1995d), _T. petchii_ (Hywel-Jones, 1997a), _T. pruinosa_ (Hywel-Jones, 1997b; Petch, 1932a), and _T. siamensis_ (Hywel-Jones, 1995d). _Cordycepioideus bisporus_, a termite pathogen, has also been shown to produce a _Hirsutella_ anamorph (Ochiel et al., 1997). In many cases, the _Hirsutella_ anamorph occurs on the developing sexual stroma. Many of these connections have yet to be confirmed through cultural studies.

_Hirsutella jonesii_ and _H. cryptosclerotium_ (Fernández-Garcia and Evans, 1990; Nag Raj and George, 1962; Evans and Samson, 1982a) produce a bulbil-like synanamorph that appears to serve as a resting spore. Two _Hirsutella_ species have unexpected synanamorphs: _Hirsutella pichilinguensis_ with its
Tetracrium-like synanamorph (Evans and Samson, 1986a), and Hirsutella petchabunensis with a Helicoma-like synanamorph (Hywel-Jones et al., 1998).


_Type species:_ Hymenostilbe muscaria Petch.


_Diagnosis:_ Colonies slow-growing, sporulation rarely seen in culture. Sporulation on the host synnematous. Conidiomata terete, compact, bearing phialidic conidiogenous cells in a hymenium-like layer. Conidiogenous cells polyblastic, irregularly short cylindrical to clavate, producing near the apex several to many slender necks or denticles, each producing a single conidium. Conidia one-celled, clavate to ellipsoid, hyaline, smooth-walled and dry.

About 13 species of Hymenostilbe are known; all are pathogens of arthropods. Some anamorphs referable to Hymenostilbe have not been named (Hywel-Jones, 1995e). Mononematous states are unknown in Hymenostilbe. To date the species have been found to grow only poorly in culture; Hywel-Jones (1996a) observed the production of synnemata but no sporulation in several strains. Repetitive conidial germination has been reported in Hymenostilbe ventricosa Hywel-Jones (1995a). The species of Hymenostilbe have not been comprehensively revised, but several partial treatments exist (Mains, 1950b; Hywel-Jones, 1995a,c,e; Samson and Evans, 1975). No Hymenostilbe species has been used as an agent of biological control.

_Hymenostilbe_ potentially intergrades with _Hirsutella_, another synnematous entomogenous genus characterized by its basally subulate conidiogenous cells that form a discontinuous layer on the synnemata, or on mononematous mycelium. _Hirsutella lecaniicola_ (Jaap) Petch, for example, has extensively polyphialidic conidiogenous cells and conidia lacking a mucous coat, and has been included by some authors in Hymenostilbe. _Akanthomyces_ differs from Hymenostilbe in its catenulate conidia and monoblastic phialides, but the older literature confounds these two genera (Petch, 1933, 1944; Mains, 1950b). The conidiogenous cells of _Granulomanus_ species are similar, but all known _Granulomanus_ species have _Gibellula_ synanamorphs.

Teleomorphs are known for some species; in some the anamorph is found on the developing sexual stroma. _H. dipterigena_ is the anamorph of _Cordyceps dipterigena_ (Brady, 1979a); _H. formicarum_ is the anamorph of _C. lloydii_ (Evans and Samson, 1984); _H. nutans_ is connected to _C. nutans_ (Hywel-Jones, 1995c; Petch, 1931), _H. sulfurea_ to _C. lutea_ (Samson et al., 1982), and _H. muscaria_ was suspected by Petch (1931) to be the anamorph of _C. forquigioni._

*Lectotype species:* *Isaria farinosa* (Holm: Fr.) Fr.

This genus was used in the past to accommodate many insect pathogens, particularly those that produce synnemata, as well as many other phylogenetically diverse fungi. Species lately assigned to *Paecilomyces*, *Hirsutella*, *Gibellula*, and others were often first described as *Isaria* species. Issues related to typification resulted in the disuse of the name *Isaria* in the twentieth century, but recent investigations suggest the name may be validly applied to the group of species now classified as *Paecilomyces* section *Isarioidea* (Samson, 1974). The precise circumscription and application of *Isaria* have not been clearly defined at this time.


*Type species:* *Lecanicillium lecanii* (Zimmerm.) Zare & W. Gams.

*Known teleomorphs:* *Cordyceps*, *Torrubiella*.

*Diagnosis:* Colonies fast-growing, white to cream, frequently forming octahedral crystals in the medium. Conidiophores little differentiated from the subtending hyphae, commonly arising from aerial hyphae, initially erect with one or two whorls of phialides, becoming prostrate and bearing large numbers of phialide whorls or single phialides. Phialides aculeate, with conidia accumulating at the tips in bundles, stacks, or chains. Short, flask-shaped, rapidly collapsing phialides which bear single conidia are present in some isolates. Conidia single-celled, hyaline, ellipsoid to falcate. Lacking dictyochlamydospores.

There are about 16 species of *Lecanicillium*; most are entomogenous or fungicolous; nematophagous species are treated by Gams and Zare (see Chapter 2). A *Lecanicillium* anamorph is formed by *Cordyceps militaris* (Zare and Gams, 2001a,b); *Torrubiella alba* was reported to be the teleomorph of *L. aranearum* (= *Engyodontium arachnophilum*) by Petch (1932a); and *Torrubiella confragosa* is the teleomorph of *L. lecanii* (Evans and Samson, 1982b). *L. tenuipes* (= *Engyodontium aranearum*) is a pathogen of spiders. Its long-legged hosts have often been misidentified as opilionids (daddy long legs or harvestmen), but Cokendolpher (1993) has determined that they are true spiders of family Pholcidae.

This segregate of *Verticillium* was proposed for species resembling *L. lecanii* (= *Verticillium lecanii*) (Zare and Gams, 2001a,b). The latter species in the broad sense has been evaluated as a biocontrol of both insects and plant parasitic fungi (Rombach and Gillespie, 1988; Rao and Pavgi, 1977, Evans and Samson, 1986b; Gillespie, 1986; Allen, 1982). Recent work (Zare and Gams,
2001a; Mor et al., 1996) has helped to refine the limits of *L. lecanii* with three similar species previously treated as *L. lecanii: L. muscarium, L. longisporum,* and *L. nodulosum.* Interpretation of the previous biocontrol literature on *V. lecanii* in light of the improved taxonomy may prove difficult unless past authors preserved voucher specimens or cultures.


*Type species: Metarhizium anisopliae* (Metschn.) Sorokin.

*Known teleomorphs: Cordyceps.*

*Diagnosis:* Conidiophores branching in a candelabrum-like fashion, forming a discontinuous hymenial layer. Conidiogenous cells cylindrical to clavate, without a neck. Conidiogenesis enteroblastic. Conidia single-celled, dry, catenulate, most often in shades of green. In culture and often on the host the conidia are produced in long chains that cohere laterally, forming compact, prismatic columns. Insecticolous.

*Metarhizium* species have been widely used for biological control of insect pests. This genus differs from *Nomuraea* chiefly in the compact conidiophores that form a hymenial layer. Five species are known; all are insect pathogens. *Metarhizium anisopliae* (Brady, 1979b) and *M. flavoviride* (Gams and Rozsypal, 1973; Rombach et al., 1986b) are the best known. *Cordyceps taii* was described by Liang et al. (1991) with a *Metarhizium taii* anamorph, but the latter is very similar to and perhaps conspecific with *M. anisopliae*.

Although the genus is well defined, the delimitation of *Metarhizium* species is difficult because of the extensive variation in host range and conidial size and color. Below the species level, many varieties have been described to accommodate groups with different conidial sizes, host preferences, DNA sequence characteristics, and cold tolerance (Rombach et al., 1986b; Driver et al., 2000; Tulloch, 1976; Rath et al., 1996). Driver et al. (2000) used ribosomal sequence data and random amplified polymorphic DNAs (RAPDs) to distinguish among species and clades. They described a number of new varieties to reflect evolutionary groups that differed in the sequence of the internally transcribed spacer region and showed correlated differences in conidial morphology. Some of these groups appear to be relatively host-restricted, although more study is needed. The varieties identified by Driver et al. (2000) are unfortunately not diagnosable without molecular data.


*Type species: Microhilum oncoperae* H. Y. Yip & A. C. Rath.

*Known teleomorph: Cordyceps oncoperae* P. J. Wright.
**Diagnosis:** Conidiophores verticillate, densely branching. Conidiogenous cells with bottle-shaped base; apex extending into a short rachis with conspicuous, conical denticles. Conidia single-celled, hyaline, subglobose, dry, with a basal “skirt” or hilum.

The unspecific genus *Microhilum* recalls *Beauveria* but lacks significant basal inflation of the conidiogenous cell. *M. oncoperae* has so far been reported only from Tasmania, Australia, on hepialid larvae (Lepidoptera). Morphological and molecular data indicate that *Microhilum* is closely allied to *Beauveria* (Sung et al., 2001; Gams and Zare, 2001). It also resembles the unspecific genus *Paraisaria*. The teleomorph connection to *Cordyceps oncoperae* was inferred from the co-occurrence of the latter species with *M. oncoperae* (Wright, 1993), and has not been confirmed through cultural or molecular study.


*Type species:* *Neotyphodium coenophialum* (Morgan-Jones & W. Gams)

*Glenn,* A. E. Glenn, C. W. Bacon & Hanlin.

*Known teleomorphs:* *Epichloë, Dussiella, Atkinsonella.*

*Diagnosis:* Colonies white to cream, slow-growing. In nature, growing as an endophyte or epiphyte of grasses, sometimes forming a small sporodochium on the host surface. Conidiophores micronematous. Conidiogenous cells aculeate, solitary or occasionally verticillate, arising at right angles to the subtending hyphae, sometimes lacking a basal septum, forming conidia enteroblastically. Conidia one-celled, ellipsoid to cylindrical, sometimes curved. Parasites of grasses.

This important genus accommodates the *Acremonium*-like anamorphs of *Epichloë* and allied genera. Most were classified as species of *Acremonium* section *Albolanosa* prior to the revision by Glenn et al. (1996), and may be found in recent literature under those names.

All known species in this group live as endophytes or epibionts on diverse grass species (Poaceae). They have been reported from over 100 grass genera (White, 1987). Some cause severe stunting or choke; others do not affect the morphology of their hosts. Toxins produced by some of these fungi have striking effects on organisms that interact with infected grasses, including grazing horses and cattle, feeding insects, and plant competitors. The interesting ecology of this interaction is discussed in Chapter 4.

The conidia are infective in some species (White et al., 1996), but their primary role appears to be to act as gametes in heterothallic species of *Epichloë* and *Dussiella* (White, 1993), and in some species are transferred by apparently mutualistic insects that act as “pollinators” (Bultman et al., 1995).
Some *Neotyphodium* forms have arisen from hybrid origin and appear obligately asexual (Schardl et al., 1994; White and Huff, 1996; Kuldau et al., 1999).

*Dussiella* (= *Echinodothis*) forms an unnamed anamorph that is referable to *Neotyphodium* and in which the conidia usually have a median septum (White, 1993). *Atkinsonella* forms a *Neotyphodium* state in addition to an *Ephelis* synanamorph (Rykard et al., 1984). Species of *Simplicillium* are morphologically similar, but lack the endophytic habit. A partial key to species was provided by White and Morgan-Jones (1987).


*Type species*: *Nomuraea rileyi* (Farlow) Samson.

*Known teleomorphs*: *Cordyceps*.

*Diagnosis*: Colonies slow-growing, developing a green or lilac tint from the accumulation of conidia. Conidiophores erect, usually simple or sparingly branched. Conidiogenous cells short flask-shaped phialides tapering to a short, narrow neck, produced in compact whorls at several levels on the conidiophore. Conidia one-celled, hyaline, dry, adhering in short chains.

*Nomuraea* species resemble those of *Metarhizium* but differ in having longer conidiophores, with a central axis bearing many levels comprised of compact whorls of conidiogenous cells on short stalks. The genus was reviewed by Kish et al. (1974); Samson (1974), and Tzean et al. (1993). The latter authors provide a comparison of known species.

Five species of *Nomuraea* are currently accepted. Two species, *N. rileyi* and *N. atypicola*, are relatively frequent: *Nomuraea rileyi* is a pathogen of noctuid larvae worldwide (Lepidoptera: Noctuidae) that is easily recognized by its leaf-green conidia. This species has been exploited for biocontrol of crop pests in several countries (Ignoffo and Boucias, 1992). It often grows as yeastlike cells in artificial culture; no teleomorph is known. *N. atypicola* is a pathogen of trapdoor and other spiders and produces lavender-colored conidia (Evans and Samson, 1987; Coyle et al., 1990; Greenstone et al., 1987). It may grow mononematously or form robust synnemata, depending on the location of its host with respect to the substrate, and sometimes produces a *Cordyceps cylindrica* teleomorph (Evans and Samson, 1987; Mains, 1954; Hywel-Jones and Sivichai, 1995). *N. viridula* and *N. cylindrosporae* form green conidia and are pathogens of cicadas known from Taiwan (Tzean et al., 1997). *N. anemonoides* also has green conidia (Hocking, 1977); it was described from soil but can parasitize some lepidopteran insects (Ignoffo et al., 1989).

*Type species:* *Paecilomyces farinosus* (Holm.: Fr.) Brown & Smith.

*Known teleomorphs:* *Cordyceps*, *Torrubiella*.

*Diagnosis:* Colonies medium- or fast-growing, usually appearing fluffy or powdery. Conidiophores erect, simple or with complex whorled branching structure. Conidiogenous cells short flask-shaped phialides tapering to a short, narrow neck. Conidia one-celled, hyaline, dry, adhering in long chains.

*Paecilomyces* is a diverse genus that is known to be polyphyletic. The type species, *P. variotii*, is affiliated with the ascomycetous order Eurotiales. Species allied to the Clavicipitaceae fall into *Paecilomyces* section *Isarioidea* Samson, a group characterized by the generally more complex conidiophores, and frequently with an insect-parasitic habit. In future this group of about 30 species will likely be considered in *Isaria* (see further discussion under *Isaria*). The genus was last monographed by Samson (1974), and a modern revision is needed.

Few teleomorph connections have been demonstrated for *Paecilomyces*. *Paecilomyces farinosus* was once erroneously considered the anamorph of *Cordyceps militaris* (Tulasne, 1857). More recently, *P. farinosus* has been suggested to be the anamorph of *Cordyceps memorabilis* (Pacioni and Frizzi, 1978). Hywel-Jones (1993) demonstrated that *Torrubiella luteorostrata* is the teleomorph of *P. cinnamomeus*. The latter species also produces an *Acremonium*-like synanamorph in culture (Hywel-Jones, 1993), and this has since been found in field-collected material (N. L. Hywel-Jones, personal communication).

Several species of *Paecilomyces*, including *P. farinosus*, *P. fumosoroseus*, *P. tenuipes*, and *P. lilacinus*, have been developed as biocontrol agents of insects and other invertebrates. *Paecilomyces lilacinus*, a nematode pathogen (Esser and El-Gholl, 1993; Bissett, 1979), may cause opportunistic infection in humans (Blackwell et al., 2000; Itin et al., 1998).


*Type (and only) species:* *Paraisaria dubia* (Delacroix) Samson & Brady.

*Known teleomorph:* *Cordyceps gracilis*.

*Diagnosis:* Colonies slow-growing, typically white to pale yellow, becoming setose with synnemata. Conidiomata synnematus, pale to chrome yellow, loose and feathery, bearing conidiogenous cells over the upper surface. Conidiophores verticillate, densely branching. Synnematus and mononematous conidiogenous cells phialidic, with a subcylindrical base tapering gradually into a narrow neck.
The conidiogenous cells often become polyblastic through sympodial development of new necks. Conidia single-celled, hyaline, narrowly fusiform, and produced in slimy heads. Insecticolous.

This unspecific genus is based on the anamorph of Cordyceps gracilis, a pathogen of beetle larvae (Brady, 1984; Samson and Brady, 1983). Its gross morphology and conidiophore branching recall species of Paecilomyces and Syngliocladium, but the conidiogenous cells are polyblastic.

7.27. Plesiospora Drechsler, Sydowia 24:174, 1971

Type species: Plesiospora globosa Drechsler.
Known teleomorphs: None.
Diagnosis: Cultural characteristics unknown. Conidiogenous cells basally swollen phialides which develop inside the body of their host; the narrow necks protrude and produce conidia externally in small slime balls. Conidia single-celled, globose, hyaline, smooth.

This unspecific genus parasitizes nematodes and is discussed further by Gams and Zare (see Chapter 2). Despite the unusual internal production of the phialides (Glockling and Yamada, 1997; Dreschsler, 1971), the conidiogenous structures closely recall Harposporium and Tolypocladium.


Type species: Pochonia chlamydosporia (Goddard) Zare & W. Gams.
Known teleomorphs: Cordyceps.

This genus name was recently resurrected by Zare et al. (2001) for the nematophagous species previously assigned to Diheterospora Kamyschko, an invalidly published generic name. Pochonia species are primarily pathogens of cyst nematodes, and most closely resemble species of Rotiferophthora, which parasitize rotifers. Cordyceps chlamydosporia H. C. Evans, a pathogen of mollusc egg sacs, is the teleomorph of P. chlamydosporia, a fungus best known as a pathogen of cyst nematodes (Zare et al., 2001). This novel connection hints at undiscovered heteroxenous life cycles in this group. Readers should refer to Chapter 2 by Gams and Zare, and Zare et al. (2001) for more information and a key to accepted species of Pochonia.

*Type species*: *Polycephalomyces formosus* Kobayasi.

*Known teleomorphs*: *Berkelella, Cordyceps*.

*Diagnosis*: Conidiomata branching synnemata, pale white to brown, bearing multiple slimy conidiogenous heads. Conidiogenous cells enteroblastic, verticillately branching, aculeate. Lateral (intercalary) conidiogenous cells may also be present. Conidia produced in slime, ellipsoid to subglobose. Fungicolous, myxomyceticolous, or insecticolous.

Accurate determination of teleomorphs in this genus has been obstructed by their apparent occurrence as parasites on various *Cordyceps* species. Some species have been described as insect parasites, others as mycoparasites (Seifert, 1985); their life cycles merit more careful study. *P. tomentosum*, a parasite of myxomycete sporangia and aethalia (Ing, 1976; Rogerson and Stephenson, 1976), has been tentatively linked to the clavicipitaceous fungus *Berkelella stilbigera* (Seifert, 1985; Rossman et al., 1999). It may merit generic distinction, as it differs from other *Polycephalomyces* species in its myxomyceticolous habit, and in the presence of ornamented sterile cells on the stipe of the synnema (Seifert, 1985).


*Type species*: *Pseudogibellula formicarum* (Mains) Samson & Evans.

*Known teleomorphs*: *Torrubiella pseudogibellulae*.

*Diagnosis*: With pale, terete synnemata arising from the body of an arthropod host. Conidiophores produced on the synnemata and on the host body, verruculose, terminating in a small, subglobose, *Aspergillus*-like vesicle. The cylindrical conidiogenous cells arise from one or two series of subglobose branches arising from the vesicle. Conidia produced singly from multiple loci surrounding the upper part of the conidiogenous cell. Conidia one-celled, ellipsoid to apiculate, dry.

This unispecific genus grossly resembles *Gibellula* but the inflated heads of the conidiophores bear *Hymenostilbe*- or *Granulomanus*-like polyblastic conidiogenous cells. The single species, *Pseudogibellula formicarum*, is a pathogen of ants (Samson and Evans, 1973; Papierok and Charpente, 1982), and is associated with a *Torrubiella pseudogibellulae* teleomorph (Samson et al., 1989).

*Type species*: *Pseudomeria mucosa* Barron.

*Known teleomorphs*: None.

*Diagnosis*: Conidiophores simple, septate. Conidiogenous cells integrated, giving rise to a short rachis just below a septum. Conidiogenous loci originate as narrow necks which develop into a slender sympodial rachis recalling that of *Beauveria*. Conidia globose, with a mucous hapteron.

A single species of *Pseudomeria* is known. It was described as a pathogen of rotifers by Barron (1980) and has not been reported since. Its morphology suggests its affinity to the Clavicipitaceae, but this has not been confirmed by molecular studies, nor through a link with a teleomorph. It is most similar to the genus *Drechmeria*, but differs in the sympodial proliferation of the conidiogenous cell.


*Type species*: *Rotiferophthora globispora* Barron.

*Known teleomorphs*: None.

*Diagnosis*: Slow-growing in culture. Conidiophores verticillately or singly branched. Phialides aculeate; intercalary phialides frequently present as slender necks. Conidia variously shaped and typically containing a single guttule, produced in small, slimy heads. Flattened, dark, dictyochlamydospores present.

Species of *Rotiferophthora* are pathogens of bdelloid rotifers (phylum Rotifera) in freshwater aquatic habitats. Hosts become infected when conidia are ingested and lodge in the buccal apparatus. *Rotiferophthora* was first described by Barron (1991), and additional species were added by Glockling and Dick (1994a) and Glockling (1998). About two dozen species are now included. *Rotiferophthora* differs from *Pochonia* in having shorter, flask-shaped conidiogenous cells and occasional intercalary phialides, flattened dictyochlamydospores, and a distinct mucous sheath surrounding the conidia (Zare et al., 2001). *Rotiferophthora* is discussed further in Chapter 2.


*Type species*: *Simplicillium lanosoniveum* (van Beyma) Zare & W. Gams.

*Known teleomorphs*: Torrubiella.

*Diagnosis*: Conidiophores prostrate and little-differentiated. Conidiogenous cells phialides, arising singly from aerial hyphae, aculeate with
a narrow tip. Conidia adhering in globose slimy heads or imbricate chains. Occurring mainly on fungi and insects.

*Simplicillium* was erected as a segregate of *Verticillium* by Zare and Gams (2001a). The name aptly reflects its simple structure. It recalls *Lecanicillium*, from which it differs in having less complex conidiophores, and mononematous *Hirsutella* species, although conidiogenous cells of the latter taper more abruptly at the neck and bear conidia in conspicuous mucous sheaths.

*S. lanosoniveum* is a parasite of rust fungi (Zare and Gams, 2001a), and may have been confused in the past with *Lecanicillium lecanii*. *S. lamellicola* (= *Verticillium lamellicola*) has also been reported as a parasite of rust fungi, and may sometimes cause a disease of cultivated *Agaricus* mushrooms (Van Zaayen and Gams, 1982).


*Type species:* *Sorosporella agrotidis* N. Sorokin.

*Known teleomorphs:* *Cordyceps variabilis*.

*Diagnosis:* Chlamydospores thick-walled, single-celled, hyaline to brick-red in mass, subglobose, usually aggregated within the body of the host. A *Syngliocladium* synanamorph may also be present on the host or in culture.

Speare (1917, 1920b) provided a detailed description of *S. uvella* and concluded that it was probably an anamorph of “*Cordyceps* or an allied type.” At least one distinct species attacks orthopteran insects (Shah and Evans, 1997; Welling et al., 1995; Shah, 1993; Shah et al., 1994; Pendland and Boucias, 1987) and Speare (1917, 1920b) described a species from a lepidopteran. Only a few species have been described, probably because of the scarcity of characters that might be used to differentiate them. Hodge et al. (1998) suggested they are better distinguished by their *Syngliocladium* synanamorphs. No comprehensive identification resources exist for *Sorosporella* species.


*Type species:* *Sphacelia segetum* Léveillé.

*Known teleomorphs:* *Claviceps*.

*Diagnosis:* Conidiomata sporodochial, sometimes becoming involute with age, arising from infected inflorescences of the grass hosts, or on the apices of ergot sclerotia. Conidiophores branching verticillately, *Gliocladium*-like, with several levels of branches. Conidiogenous cells
tapering to a narrow tip, producing many conidia enteroblastically in copious slime (honeydew). Conidia ovoid to ellipsoid, sometimes reniform or apiculate.

*Sphacelia* species are anamorphs of *Claviceps* species, and cause ergot diseases of grasses (Langdon, 1954; Pazoutová, 2001). The conidia are exuded on grass inflorescences in a sugary slime called honeydew (Mower and Hancock, 1975), to which insects are strongly attracted. Conidia may germinate repetitively in some species (Frederickson et al., 1989). Like other members of the Clavicipitaceae, *Sphacelia* species produce toxic alkaloids that can have serious physical and psychological effects on grazing animals and humans (Matossian, 1989; Bove, 1970).

Although the characteristics of the teleomorph are important in species diagnosis and identification, Loveless (1964) emphasized the utility of anamorphic characters in separating the species and provided a table comparing conidial groups. Over 30 species have been described, but no comprehensive identification resources based on the anamorph have been developed. San et al. (1997) provided a key to species known from Mexico.


*Type species*: *Syngliocladium aranearium* Petch.

*Known teleomorphs*: *Cordyceps variabilis*.

*Diagnosis*: Mononematous or synnematous with loosely fasciculate synnemata. Conidiophores *Gliocladium*-like, bearing terminal clusters of tapering conidiogenous cells, or forming uncinate conidiogenous cells singly or in pairs on repent hyphae. Conidiogenesis enteroblastic, phialidic, conidia produced in copious slime. Conidia single-celled, ovoid to cylindric, hyaline or orange in mass. A chlamydosporic *Sorosporrella* synanamorph may be present. Insecticolous.

Petch (1932b) described *Syngliocladium* for *S. aranearium* Petch, a spider pathogen found in Britain; this species has not been reported since that time. The name *Syngliocladium* has most commonly been applied to the synanamorph of *Sorosporrella uvella*, which forms thick-walled resting spores inside the bodies of its hosts. Not all *Syngliocladium* species produce *Sorosporrella* synanamorphs. Hodge et al. (1998) extended the genus and provided a discussion of known *Syngliocladium* species. *Cordyceps variabilis* produces an anamorph attributable to *Syngliocladium* (Hodge et al., 1998).

*Type species*: None designated.  
*Known teleomorphs*: None.

The genus *Tilachlidiopsis* has been used to accommodate insect pathogenic species, however, no type species for the generic name was selected by Keissler (1924). Lectotypification of the genus with one of the two original species, *T. racemosa* or *T. hippotrichoides*, will determine the application of the generic name and permit an appropriate description. *T. racemosa* has thallic conidial ontogeny, and Stalpers et al. (1991) have shown it to be the anamorph of the mushroom *Collybia racemosa*. *T. hippotrichoides* may be clavicipitaceous.

Seven species have been assigned to *Tilachlidiopsis*. Some of these appear to be clavicipitaceous. The latter include *Stilbella burmensis* (Mains) Samson & Evans, *Stilbella dolichoderinarum* Samson & Evans, *Stilbum buquetii* Mont. & C. Robin (Seifert, 1985), *Isaria nigra* Yaskushiji & Kumazawa, *T. scarabaei* L. S. Olive, and *T. catenulata* Papierok & Charp. None of these species is well known, and few have been reported from more than a few specimens. *T. piptadeniae* Bat. & H. Maia and *T. brasiliensis* C. Ram & A. Ram were excluded from the genus by Papierok and Charpentie (1982). No identification resources exist for this group, and taxonomic revision is needed.


*Type species*: *Tolypocladium inflatum* W. Gams, nom. cons.  
*Known teleomorphs*: *Cordyceps*.

*Diagnosis*: Colonies moderate to fast-growing, typically white to cream. Sporulation mononematous, conidiophores branching at right angles, reminiscent of *Trichoderma* in structure, producing conidiogenous cells terminally and laterally, singly or whorled, at right angles to the conidiophores. Conidiogenous cells phialides, with a flask-shaped base that tapers into a narrow neck. The neck is often bent away from the axis of the conidiogenous cell. Conidia single-celled, hyaline, smooth, typically globose to cylindrical, produced in slimy heads.

Von Arx (1986) considered *Tolypocladium* a synonym of *Beauveria* on the basis of their resemblance in culture and the basally inflated conidiogenous cells. His conclusions have not been accepted by most authors, and molecular and biochemical characters do not support his arguments (Todorova et al., 1998; Mugnai et al., 1989; Kadlec et al., 1994). *Harposporium* is very similar, but has infrequently branched conidiophores, unusually shaped spores, forms a darker mycelium in axenic culture, and typically parasitizes nematodes and rotifers.
T. trigonosporum Barron, a rotifer pathogen, appears somewhat intermediate between Tolypocladium and Harposporium. Culcinomyces is also very similar to Tolypocladium. C. parasiticus (Barron) Sigler (Sigler et al., 1987) was originally described as a Tolypocladium. The nematode pathogen T. balanoides ( = Verticillium balanoides) now typifies the genus Haptocillium (Gams and Zare, 2001). The rotifer and nematode pathogenic species are discussed further in Chapter 2.

Insect pathogenic species of Tolypocladium were reviewed by Samson and Soares (1984); they are especially predominant pathogens of nematoceran flies (Diptera: Nematocera). The type species of Tolypocladium, T. inflatum (the name T. niveum was formerly applied to this fungus, but T. inflatum has been nomenclaturally conserved and must be used instead) is frequently isolated from soils in the temperate zone. Its affiliation with the Clavicipitaceae was unknown until it was linked to Cordyceps subsessilis—a pathogen of scarabaeid beetles (Hodge et al., 1996). T. inflatum is a source of the immunosuppressant drug, cyclosporin (Borel and Kis, 1991), and of various efrapeptins (Krasnoff and Gupta, 1992), that show insecticidal activity. Samson and Soares (1984) felt the frequency of Tolypocladium species in soils suggested an alternate, soil-dwelling host, but so far no data support this hypothesis.


Lectotype species: Ustilaginoidea virens (Cooke) Takahashi.
Known teleomorphs: Claviceps-like, but unnamed.
Diagnosis: Forming a dark, hard sclerotium-like stroma around the ovaries of their grass hosts. Conidia arising from short lateral denticles from undifferentiated mycelium, forming balls of dark conidia. Conidia one-celled, verruculose, dry, dark olive green to black in mass, subglobose. In culture, the conidia may germinate to form small heads of smaller, paler, smoother ameroconidia (Brefeld, 1895; Mulder and Holliday, 1971).

Ustilaginoidea species cause false smut diseases on various grasses, including rice and maize. Their parasitism of the host’s ovaries and the formation of a sclerotium-like structure recalls species of Claviceps (Sphacelia anamorphs). The dark, roughened conidia are unique for the family Clavicipitaceae, and closely resemble teliospores of true smuts in the order Ustilaginales, with which they have often been confused. About 20 species have been reported; almost all are very poorly known. The best known is U. virens, cause of an economically important false smut of rice, but few details of its life history have been studied (Mulder and Holliday, 1971; In et al., 1984; Fujita et al., 1989).
The perithecial ascomata resemble those of *Claviceps* (In et al., 1984), and arise from a similar sclerotal mass, but no teleomorph name is in current use for this state. Diehl (1950) placed *Ustilaginoidea* and *Claviceps* in sister tribes, which differed chiefly in their anamorphic states. As pointed out by White et al. (2000), further work is badly needed to elucidate life cycles and taxonomy in this group.


The form genus *Verticillium sensu lato* is polyphyletic, including species affiliated with several ascomycete orders. Most species allied with the Clavicipitaceae fall into *Verticillium* sect. *Prostrata* W. Gams. Their simple morphology has confounded past efforts to divide it into natural groups. Recently, Zare and Gams undertook an ambitious redistribution of species of *Verticillium* sect. *Prostrata* into several segregate genera, based on morphological and molecular data (Zare et al., 2000, 2001; Sung et al., 2001; Gams and Zare, 2001; Zare and Gams, 2001a,b; Zare and Gams, 2001c). These include *Simplicillium*, *Lecanicillium*, *Pochonia*, and *Haptocillium* (q.v.). Species of *Rotiferophthora* are similar. Only a few potentially clavicipitaceous species now remain in *Verticillium*, and it is expected that these will be transferred to more appropriate genera as confirmatory data arise.

8. **UNNAMED CLAVICIPITACEOUS ANAMORPHS**

Although most known clavicipitaceous anamorphs are readily referred to the above genera, a handful exhibit novel combinations of characters and have not been formally classified.

The recently described genus *Hyperdermium* (Sullivan et al., 2000) exhibits a novel anamorph that the authors compared to the Hypocrealean anamorph, *Cylindrocarpon*. It produces simple aculeate phialides and the conidia are multi-septate and accumulate in a microsclerotium-like mass which apparently functions as a diaspore. Sullivan et al. (2000) inferred a relationship between *Hyperdermium* and *Cordyceps militaris* based on molecular data. Further taxon sampling is needed to elucidate the nearest relatives of *Hyperdermium*.

Two atypical synanamorphs have been described for *Hirsutella* species. Evans and Samson (1986a) described an unusual synanamorph of *Hirsutella pichilinguensis* Evans & Samson. It produced pale brown, clavate, distoseptate conidia on the body of the insect host, and on the apex of *Hirsutella* synnema. The synanamorph was not separately named, but was described as *Tetracrium*-like (Evans and Samson, 1986a). Hywel-Jones et al. (1998) described a *Helicoma*-like synanamorph of *Hirsutella petchabunensis*, a pathogen of Lepidoptera in Thailand. Both *Helicoma* and *Tetracrium* are anamorphs typical
of the family Tubeufiaceae (Pleosporales). These unusual forms recall another Hirsutella synanamorph, Desmidiospora, in their thick-walled dark conidia. They differ strikingly from all other known clavicipitaceous anamorphs, yet their insect-pathogenic habit and Hirsutella synanamorphs suggest a relationship with the Clavicipitaceae.

Most clavicipitaceous anamorphs described as “Acremonium-like” have recently been transferred to the genus Neotyphodium, which accommodates the anamorphs of Epichloe and some related grass endophytes. Others have been included in genera recently segregated from Verticillium (Gams and Zare, 2001; Zare and Gams, 2001a; Zare et al., 2001). The Acremonium-like anamorphs of Neobarya Lowen (Eriksson and Hawksworth, 1986) have not been fully described, and current data are insufficient to suggest an appropriate generic placement.

Suh et al. (2001) have demonstrated that several endosymbiotic yeasts of some plant hoppers are members of the Clavicipitaceae. The origins of these very reduced forms are uncertain, but molecular data points to a relationship with insect pathogenic species including the holomorph genus Cordycepioideus and anamorph genus Hirsutella. The yeasts are transmitted from parent to offspring within the egg, and have not been axenically cultured. Only three planthopper yeasts have been characterized to date; no name has been applied.

Molecular data may yet reveal more anamorphs that have not yet been connected with the Clavicipitaceae. Our poor state of knowledge about tropical species in particular suggests that many new anamorph forms remain to be discovered.

REFERENCES


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The Plant-Infecting Clavicipitaleans

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1. INTRODUCTION

Diehl (1950) listed three subfamilies within the Clavicipitaceae: Oomycetoideae, Clavicipitoideae, and Cordycipitoideae. Within these three subfamilies he listed a total of 24 plant-infecting clavicipitalean genera, of which nine genera could be synonymous. Rogerson (1970) included 18 plant-infecting clavicipitalean genera, five of which he considered synonymous with other genera. He did not, however, include genera whose teleomorphic connections were either unknown or not well understood. Eriksson et al. (2001) included 17 plant-infecting clavicipitalean genera within the family. However, like Rogerson (1970) Eriksson et al. did not include the form genera Munkia, Neomunkia, and Ustilaginoidea, which are believed to be members of the Clavicipitaceae based on stromal morphology (Diehl, 1950) and molecular data (White et al., 2000). Teleomrophic affinities of these form genera have been hypothesized (Petrak, 1947; Hashioka et al., 1951) but never determined with any certainty.

The aim of this chapter is to outline all known plant-infecting clavicipitalean genera. The data included in this chapter are based on original descriptions and the investigations that followed. Recent taxonomic studies, employing DNA sequence analyses, have shed light on the placement of certain
genera whose membership within the family has been in question or unknown. Questions regarding the placement of many of the genera included in this chapter remain. Rogerson (1970) wrote, “Perhaps this review and presentation of keys for identification of genera will aid and stimulate the search for fresh material and restudy of the species, particularly those on which genera have been based.” The goal of this treatment is the same as that of Rogerson over 30 years ago.

2. TAXONOMIC TREATMENT

2.1. Key to the Plant-Infecting Genera of Clavicipitaceae

1a. Conidia produced pleurogenously; no observable teleomorphic state ................................................................. 2
1b. Not as above........................................................................4
2a. Conidia echinulate; stroma replaces ovules .......... *Ustilaginoidea*
2b. Conidia smooth, stroma produced along stems .............3
3a. Conidiophores produced in irregular acervulus-like structures .................................................. *Neomunkia*
3b. Not as above..................................................................... *Munkia*
4a. 2 conidial states....................................................................5
4b. 0–1 conidial states...............................................................6
5a. Occurs on Bambuseae.................................................. *Loculistroma*
5b. Occurs on various grasses (not Bambuseae)............... *Atkinsonella*  (see *Balansia* subgenus *Eubalansia*)
6a. Single perithecium in stipitate stroma............... *Microstelium*
6b. Multiple perithecia in stroma..........................................7
7a. Perithecial stroma not stipitate ................................................14
7b. Perithecial stroma stipitate.................................................8
8a. Perithecial stroma capitate ....................................................9
8b. Perithecial stroma clavate..................................................12
9a. Stroma develops from an exposed sclerotium........ *Claviceps*
9b. Not as above..................................................................10
10a. Stroma forms choke around grass inflorescence .......... *Balansia*  (subgenus *Eubalansia*)
10b. Not as above..................................................................11
11a. Ascospores hyaline.................................................... *Neoclaviceps*
11b. Ascospores yellow to green................................. *Stereocrea*
12a. Ascospores bifusiform which are connected by a fine filament .................................................. *Phytocordyceps*
12b. Ascospores fusiform....................................................13
13a. Ascospores swell at maturity ......................... *Shimizuomyces*
13b. Ascospores not as above ............................................ *Neocordyceps*
14a. Stroma bulbous on or around stem ........................................... 15
14b. Stroma superficial on stem or leaf ........................................ 18
15a. Stroma black ....................................................................... Konradia
15b. Stroma not as above ............................................................ 16
16a. Conidia holothallic or chlamydosporic .............................. Cavimalum
16b. Conidia not produced as above ............................................. 17
17a. Mature stroma polypore-like ............................................ Ascopolyporus
17b. Stroma subglobose and white in cross-section ................ Mycomalus
18a. Mature stroma is black ...................................................... 19
18b. Stroma color not as above .................................................. 21
19a. Perithecia in 1 or 2 rows along the length of the stroma .......................................................................................... Myriogenospora
19b. Not as above ........................................................................ 20
20a. Stroma covers inflorescence ............................................ Parepichloë
20b. Stroma surrounds the culm ................................................. Balansia (subgenus Dothichloë)
21a. Perithecia develop from multiple pulvinate stromal tissue Dussiella
21b. Not as above ........................................................................ 22
22a. Numerous perithecia when mature ..................................... Epichloë
22b. Perithecia rarely present or 1–3 present per stroma Hyperdermium

3. THE GENERA
3.1. Ascopolyporus Möller

Type species: Ascopolyporus polychrous Möller.

Habit and type host: On dead culms of bamboo.

Collected from: Guadueae, Blumenau, Brazil, South America.

Figure: 1.

Description of type species: Stroma grows to approximately 4 cm in diameter, may be bright-rusty red or white to yellow; early in development conidial state covers the hypothallus, then perithecial stroma grows to cover hypothallus; perithecial stroma usually fertile only on underside of stroma; in cross section dark radiating lines and a soft aqueous center are present; stroma resembles a sporocarp of Polyporaceae, Basidiomycota. Perithecia up to 750 μm in length, narrow obclavate, crowded. Asci up to 500 × 4 μm, hyaline, 8 ascospores per ascus. Ascospores 300 × 1 μm, filiform to spiroid, hyaline, disarticulate into partspores approximately 6 μm long. Conidia 7–12 × 4–6 μm, white, oval, one- to multicelled, congregating at phialide tips.

Möller (1901) observed that the fruiting bodies that were reddish in color were always associated with star-shaped insect galleries.

The attachment of Ascopolyporus spp. reminds one of Hyperdermium and Hypocrella spp. in appearance. The hypothallus is superficial and encircles the plant stem approximately three-fourths of the way around. Like Hypocrella, there is a soft and sparsely filled location in the stroma at the attachment. These similarities may indicate that Ascopolyporus is a scale insect pathogen that is able to utilize the plant sugars after the scale insect has been consumed.

3.2. **Balansia Subgenus Eubalansia Diehl**


*Type host: Stromata on inflorescences of unknown panicoid host.*
Collected from: Pirayu, Paraguay.

Figure 2.

Description of type species: Stroma 15–20 cm long \times 2–4 mm wide, light brown to black, smooth, fibrous, white in cross section. Perithecial stroma, 1.5–3.0 mm in diameter, stipitate to sessile, hemispherical to globose, hard, papillate, dark brown to black, arising from stroma; stipe, 0.5–3 mm long \times 1.0–1.5 mm wide, scurfy. Perithecia, 220–280 \times 80–130 \mu m, obovate, immersed except for ostiolar neck, crowded. Ascii, 120–180 \times 5–6 \mu m, cylindrical, rounded apex, thickened wall, 8 ascospores per ascus, base slightly attenuated, aporaphysate. Ascospores are the length of ascus, 0.6–0.8 \mu m wide, filiform, septate, and hyaline. Conidia ephelidial, produced in cupulate conidiomata. Receptive hyphae in mounds that later develop into stipitate perithecial stromata.

Other species in subgenus: B. asclerotiaca Henn.; B. ambiens Möll.; B. clavula Berk. & Curt.; B. cyperi Edg., B. hypoxylon (Pk.) Lewis & White ( = Atkinsonella hypoxylon); B. obtecta Diehl; B. texensis (Diehl) Lewis & White ( = Atkinsonella texensis).

Several different species of Asian and American Balansia are often misidentified as B. claviceps. Balansia claviceps may be distinguished from all similar species because it is limited to the Americas, is endophytic, and produces

Figure 2 Balansia claviceps (12X).
stipitate perithecial stromata. For additional descriptive information see Reddy et al. (1998); Sprague (1950), and Saccardo (1891).

Species formally placed in the genus *Atkinsonella* are distinguished from other *Balansia* subgenus *Eubalansia* species by the occurrence of two conidial states (micro- and macroconidia). For additional descriptive data on *Atkinsonella* see Diehl (1950), Morgan-Jones and White (1989), Leuchtmann and Clay (1989), and Lewis et al. (Chapter 5, this volume).

### 3.3. *Balansia* (Subgenus *Dothichloë* Diehl)

*Type species:* *Balansia aristidae* (Atk.) Diehl = *Dothichloë aristidae*  

*Habit and type host:* Stromata encircle culm at nodes and part of adaxial leaf sheath of *Aristida purpurascens*.

*Collected from:* Auburn, Alabama, USA, North America.

*Figure:* 3.

*Description of type species:* Stromata 8–20 mm long, at first light green to pale brown, covered to varying degrees with an ephemeral ephelidial conidial state, then black, papillate, up to 30 μm thick. Perithecial stroma pulvinate (whole or fragmented) covering part or entire stroma. Perithecia 305–400 × 130–200 μm, lageniform to ovate, ostiolar necks protruding from stromal peridium. Asci 140–255 × 4–6 μm, cylindrical to subcylindrical. Ascospores 70–190 × 1–2 μm, filiform, disarticulating within ascus into partspores, 21–34 μm (Atkinson, 1894).

![Figure 3](image-url) **Figure 3** *Balansia aristidae* (left 7X and right 1X).
Other species in subgenus: *B. cyperacearum* (Berk. & Curt.) Diehl; *B. discoidea* Henn.; *B. epichloë* (Weesse) Diehl; *B. gaduae* (Rehm.); *B. hemicyprota* Diehl; *B. henningsiana* (Moell.) Diehl; *nigricans* (Speg.) White et al.; *B. strangulans* (Mont.) Diehl.

For additional descriptive data on *Balansia aristidae* see Diehl (1950) and White et al. (1997). *Balansia aristidae* is also known as the black choke disease (Sprague, 1950).

3.4. **Genus Cavimalum** Doi

*Type species*: *Cavimalum indicum* Doi.

*Habit and type host*: Stromata are found on living stems of *Bambusa* species.

*Collected from*: India, Asia.

*Description of type species*: Stroma 1.5–3.0 cm in diameter, subglobose, fleshy, gray–green, later becoming green–yellow; surface, puncate, occasionally with hisrute synnemata that project from the stroma. Stroma has an aqueous center, surrounded by the stroma peridium, 1–2 mm in diameter. Perithecia 500–600 × 200–300 μm, with 10–20 μm thick walls, obclavate to panduriform, periphysate, immersed except ostiole neck slightly protrudes from stromal surface. Asci 400–450 × 15–25 μm, cylindrical to elongate fusiform, with a small apical pore, containing 8 ascospores, unitunicate. Ascospores 300–350 × 4.5–7.5 μm, hyaline, guttulate, irregularly septate with 4–12 μm-long segments. Conidial state occasionally present on synnematal-like structures, producing aleuriospores, 5–8 × 2.5–4 μm (Doi et al., 1977). *Other species in genus*: *C. borneense* Doi et al.

This species was discovered in India while the only other species within the genus, *Cavimalum borneense*, was discovered in Borneo (Doi et al., 1977). It is unknown whether ascospores separate into part spores.

3.5. **Genus Claviceps** Tul.


*Habit and type host*: Infecting ovules of *Secale cereale*.

*Collected from*: France, Europe.

*Figure: 4.*
Description of type species: Sclerotia 5–30 mm long, oblong, curved cylindrical, spurlike, surface is purple–black and comprised of pseudo-parenchymatous cells, white in cross section. Stromata 5–25 mm long, stipitate and protruding from sclerotia; perithecial stroma is papillate from protruding ostiole necks, globose to subglobose, 1–60 stromata form per sclerotium. Perithecia 150–175 × 200–250 μm, obpyriform, imbedded. Asci 100–125 × 4 μm, hyaline, cylindrical to clavate, with 8 spores per ascus. Ascospores 50–76 × 0.6–0.7 μm, filiform, later septate, forcibly ejected. Conidial stage (Sphacelia sp.) 4–6 × 2–3 μm, elliptical, aseptate.

Other species in genus: C. africana Frederickson et al.; C. cinerea Griffiths; C. citrina Pazoutova et al; C. grohii Groves; C. junci Adams; C. nigricans Tul.; C. ranunculoides Möll.; C. rolfsii Stevens & Hall; C. tripsaci Stevens & Hall; C. zizaniae (Fyles) Pantidou.

Early in the infection a honeydew-like secretion exudes from the infected florets of grasses. Conidia may be found in this honeydew secretion. As the sclerotic structure becomes evident, production of honeydew may continue to be...
found on the sclerotia (Sprague, 1950). Sclerotia that form on grasses normally located in aquatic environments are able to float, while sclerotia forming on terrestrial grasses usually sink in water (Stäger, 1922). For additional information on *Claviceps* see Heald (1926) and Pazoutová and Parbery (1999).

### 3.6. Genus *Dussiella* Pat.

*Type species:* *Dussiella tuberiformis* (Berk. & Ravenel) Pat. = *Hypocrea tuberiformis* Berk. & Ravenel = *Echinodothis tuberiformis* (Berk. & Ravenel) Atk.

*Habit and type host:* Stromata on culms of *Arundinaria* sp.

*Collected from:* North or South Carolina, USA, North America.

*Figure:* 5.

*Description of type species:* Stromata at maturity composed of one to multiple, subglobose perithecial stromata, 4.8–7.5 mm (each lobe) in

![Figure 5](image_url)  

**Figure 5**  *Dussiella tuberiformis* (5.5x).
diameter, off-white to gray. Hypothallus radiating from stroma, white to pallid, attached superficially to host culm. Stroma first covered by a conidial hymenial layer, later perithecial stromata develop on surface. Stroma core is moist, pink to light purple, surrounded by white to yellow mycelial layers. Hyphae in the outer layers are 1.2–2.3 \mu m, while hyphae near the center of the stroma are 4.3–18.9 \mu m in diameter and contain more granules. Perithecia 820–1120 \times 103.5–128.5 \mu m, subcylindrical, with short periphyses. Perithecial development begins with an off-white mycelial network forming over portions of stroma from which cylindrical extensions form that eventually contain a single perithegium. Ascii 570–610 \times 8.7–10.7 \mu m, cylindrical, with a refractive tip, guttulate until ascospores develop, aparaphysate. Ascospores 550–570 \times 3.7–4.3 \mu m, filamentous, hyaline, multisepate, breaking into part spores, 10.8–15.8 \times 2.3–3.3 \mu m. Conidial state present as a hymenial layer over the immature stroma. Conidiogenous cells phialidic, hypha-like, gradually attenuate, 36.1–45.1 \times 2 \mu m at base to 0.5 \mu m at apex. Conidia lunate, 1-septate, first 4.0–5.0 \times 1.9–2.1 \mu m, then swelling to 6.8–8.1 \times 2.3–3.1 \mu m (White, 1993).

Other species in genus: D. gaduae (Henn. as Echinodothis gaduae); D. orchideacearum Rick; D. violacea Höhnel.

**Dussiella tuberiformis** has recently been shown to produce perithecia in culture (Lewis and White, personal communication, 2001).

### 3.7. Genus *Epichloë* (Fr.) Tul. & C. Tul.


**Habit and type host:** Stromata covering inflorescence primordia of *Dactylis glomerata*.

**Collected from:** France, Europe.

**Figure: 6.**

**Description of type species:** Stromata 32.2–62.8 mm in length (diameter dependent on diameter of host culm), around culm, enclosing inflorescence primordia, leaf primordia and leaf sheath, cylindrical, first pallid, smooth and moist in appearance, covered with hymenial (conidial) layer, later yellow to orange and verrucose with emergence of perithecia. Hymenial layer composed of hypha-like conidiogenous cells,
16.6–28.6 × 1.8–2.7 μm at base and tapering to 1 μm at apex, at or near perpendicular to stroma. Spermatia (conidia) 4.5–5.5 × 2.0–3.0 μm, hyaline, reniform to hemispherical, forming in small groups near tip of conidiogenous cell. Perithecia 274.3–313.7 × 98.1–161.9 μm at base and 108.4–137.6 μm at neck, orange to red, obpyriform to obclavate, periphysate, immersed in hymenial stroma with only the truncated ostiolar neck emergent. Asci 145.2–180.8 × 6.7–7.1 μm, cylindrical, hyaline, with apical pore through thickened tip, containing 8 ascospores. Ascospores 141.7–210.3 × 1.4–1.8 μm, filiform, hyaline, septate, ejected entire.

Other species in genus: E. amarillans White; E. baconii White; E. clarkii White, E. festucae Leuchtmann et al.

Species of Epichloë are endophytic in grasses. Epichloë typhina has been shown to be heterothallic and requires deposition of spermatia of the opposite mating type in order to produce perithecia (Bultman and White, 1997). The neotyphodial conidia serve as spermatia which are transported between stromata by Anthomyiid flies (Kohlmeyer and Kohlmeyer, 1974; Bultman and White, 1997). The conidial state of Epichloë has been classified in the anamorphic genus Neotyphodium (Glenn et al., 1996).


Type species: Hyperdermium bertonii (Speg.) White et al. = Epichloë bertonii Speg.

Habit and type host: Stromata on stems of unidentified Asteraceae.

Collected from: Costa Rica, Central America.

Figure: 7.

Description of type species: Stroma 0.5–10 cm long, yellow to orange, crustose, sometimes encircling stem. Perithecia, 200–250 × 65–80 μm,
cylindrical, rarely occur. Asci 100–160 × 8–9 μm, cylindrical to slightly fusiform, enlarged refractive apical tip. Ascospores extend the length of ascus × 1 μm, hyaline, filamentous, multisepate. Conidia first 5–7 × 1–1.5 μm, aseptate, then enlarging to 15–30 × 1.5–3 μm and up to 5 septa, cylindrical to fusiform, accumulating at phialide tips, produced enteroblastically. Phialides often possess a collarette at apex (Sullivan et al., 2000).

Other species in the genus: Hyperdermium pulvinatum White et al.

Hyperdermium bertonii is believed first to infect and necrophytize a scale insect that has parasitized the plant host. Afterward, the fungus acquires nutrients that leak from the plant’s phloem tissues through the stylet wound (Sullivan et al., 2000).

3.9. Genus Konradia Raciborski

Type species: Konradia bambusina Raciborski = Xylariopsis bambusina Tai.

Habit and type host: Stromata on culms of Melocanna sp.

Collected from: Java.

Description of type species: Stromata 1–6 × 5–7 mm, forming around entire culm or at node of the unrolling culm, black, subglobose to cylindrical, spongy, brownish hyphae cover the stroma; at maturity surface punctate with minute ochreous ostioles. Perithecia 125–160 μm in diameter, immersed, globose, ostioles slightly emergent from surface of stroma. Asci 74–80 μm long and up to 18 μm wide, clavate, with 8 ascospores per ascus. Ascospores at first filiform and yellowish, later disarticulating into numerous smooth partspores, 4.0 × 3.0 μm, which become brown to black upon dispersal (Saccardo, 1902).

Other species in the genus: K. secunda Rac.
3.10. **Genus: Loculistroma Patterson**

*Type species:* Loculistroma bambusae Patterson.

*Habit and type host:* Stromata on inflorescences of Phyllostachis spp.

*Collected from:* Hankow, China, Asia.

*Description of type species:* Stromata 10 mm long \( \times \) 2 mm across, cylindrical, carnose, subsessile, green to black. Perithecia 125 \( \times \) 100 \( \mu \)m, sub-immersed in stroma, subglobose. Asci 40–50 \( \times \) 9–10 \( \mu \)m, clavate to cylindrical, with 8 ascospores per ascus. Ascospores 22 \( \times \) 4.5–5.0 \( \mu \)m, fusiform, olivaceous, 3 septations per ascospore, arranged in a distichous formation within the ascus. Conidial states of two types: primary conidia, 14–16 \( \times \) 0.75–1 \( \mu \)m, filiform, hyaline, produced in sporodochia on conidiophores, 8.0 \( \times \) 0.5 \( \mu \)m; secondary conidia, one- to three-celled, olivaceous, Cladosporium-like.

*Other species in genus:* None.

Loculistroma bambusae has been suggested to be a synonym of Balansia take (= Aciculosporium take) (Patterson and Charles, 1910). However, due to the occurrence of two conidial states and differences in ascospore color, size, and shape, these two species do not appear to be synonymous.

3.11. **Genus: Microstelium Patouillard**

*Type species:* Microstelium kyalinum Patouillard.

*Habit and type host:* Stromata on moss and lichens growing on wood.

*Collected from:* Near Bains-Larmes, Guadeloupe, West Indies.

*Description of type species:* Subiculum composed of white, superficial, branched, slender mycelium. Perithecia 1 mm long, upright, stipitate, cylindrical, with blunt-rounded apex, violet to brown. Asci 8–10 \( \mu \)m wide, long, cylindrical, intermixed with filiform paraphyses; Ascospores disarticulating into many parts, 6–8 \( \mu \)m long. (Patouillard, 1899; Saccardo and Sydow, 1902).

*Other species in genus:* None.

Walker (1980) examined the type and could not find fungal structures. However, according to Patouillard’s original description, Microstelium kyalinum resembles Acrosporum and Barya.

3.12. **Genus: Munkia Speg.**

*Type species:* Munkia martyris Speg.

*Type host:* Stromata on branches of Chusque sp. (Poaceae).

*Collected from:* Guarapi, Brazil, South America.
Figure: 8.

Description of type species: Stroma 3–10 mm in diameter, globose to lenticular, somewhat leathery, hard when dry, irregularly applanate to rimose, gray–green with white dots (conidiomata). Conidial state: conidiophores 450–600 × 2.5 μm, densely packed together in scattered cupulate conidiomata (200–300 μm in diameter), simple, upright, fasciculate. Conidia, 2.5–3.5 μm in diameter, globose to subglobose, single gutulate, hyaline, pleurogenously produced on the distal end of the conidiophores from papillate structures (Petrak, 1947; Saccardo, 1892; Stevens, 1927).

Other species in genus: M. guaranitica Speg.; M. strumosa (Cooke) Marchionatta.

According to Petrak (1947), Spegazzini named another Munkia species, M. guaranitica, and connected it with a Mycomalus-like teleomorph. A teleomorph has not yet been linked with Munkia martyris. Stevens (1927) had described
Shropshiria chusque, which was later transferred to the genus *Munkia* by Marchionatto (1940).

### 3.13. Genus *Mycomalus* Möller

*Type species:* *Mycomalus bambusinus* Möller.

_Habit and type host:_ Stomata on culms of *Guadua taguara*.

_Collected from:_ Blumenau, Brazil, South America.

_Description of type species:_ Stroma up to 6 cm in diameter, subglobose, compressed at ends, with a dark brown infertile zone surrounded by lighter brown, papillate (ostiolar necks) fertile zone (perithecial stroma); infertile zones are exposed from the perithecial stroma where contact with the host culm is made; stroma white in cross section. Perithecia up to 2 mm from the base to ostiolar tip, immersed, obpyriform, average of 9 perithecia/cm²; ostioles appear as dark dots, white spore masses sometimes visible at ostioles. Asci up to 1 mm, hyaline, with 8 ascospores per ascus. Ascospores at first filiform, then disarticulating into partspores 30–50 \( \mu \text{m} \) long, fusiform. After exiting the ascus, ascospore septa become visible. Conidia globose, produced on short phialides directly from ascospores (Möller, 1901).

_Other species in the genus:_ None.

Möller (1901) suggested that there are originally 8 filiform ascospores in each ascus. These ascospores then disarticulate into many partspores, which then elongate and swell to become fusiform and fill up the ascus.


_Habit and type host:_ Stomata on rolled or folded leaves of *Paspalum* sp.

_Collected from:_ Alabama, USA, North America.

_Description of type species:_ Stroma 15–200 × 0.5–0.6 mm (length dependent on host), at first brown then becoming black, extending up the center of a rolled or folded leaf blade. Perithecia 260–302 × 350–400 \( \mu \text{m} \) at end of stroma including the ostiolar neck (perithecia may vary greatly in size), borne in a singular row along length of stroma; in cross section perithecia are globose to subglobose; perithecia within the row are subglobose to rectangular due to compression from neighboring
perithecia; perithecia are completely immersed, with short ostiolar necks that form a slightly papillate stromal surface. Periphyses present in ostiolar neck. Asci 180–312 × 12–23 μm, clavate when young, later becoming broadly fusiform, 8 coiling ascospores per ascus, with a dome-shaped apex (refractive tip absent), swelling beneath apex at maturity; releasing ascospores by circumsessile break in ascus at base of apical dome. Paraphyses surround the perithecial centrum but none are present among asci. Ascospores at first filiform, septate, then breaking at septations into narrowly fusoid partspores, (7)–23–45 × 1.5–2 μm. Conidial state ephelidal, occurring early in stroma development, not present after darkened stromal layer and perithecia form. Conidia 14–36 × 1.5–2.0 μm, narrow fusiform, often curved, single-celled (Luttrell and Bacon, 1977; Hanlin and Tortolero, 1990; White and Glenn, 1994).

Other species in genus: M. bresadoleana Henn., M. linearis (Rehm.) White & Glenn.

*Myriogenospora atramentosa* is the causal organism of “tangle top disease” of many grasses, in which the tips of grass blades become embedded in the stroma found on its blade or neighboring blade, causing the blade to form a loop (Diehl, 1934). The host leaf is often lighter green than healthy host tissues, and some purple coloration may appear around the stroma.

### 3.15. Genus: *Neoclaviceps* J. White, G. Bills, S. Alderman, and J. Spatafora

*Type species:* *Neoclaviceps monostipa* J. White, G. Bills, S. Alderman, and J. Spatafora

*Habit and type host:* Stromata in florets of unknown panicoid grass.

*Collected from:* Costa Rica, Central America.

*Figure:* 9

*Description of type species:* Hypothallus 1–1.5 × 2–3 mm, within ovary, with a single stipitate perithecial stroma. Stipe up to 3 mm long and 0.2–0.3 mm wide, reddish-brown, portions with scurf, whitish collar of mycelium at base. Perithecial stroma 0.75–1.0 × 0.6–0.8 mm, hemispherical, reddish-brown, containing 28–50 papillate perithecia on apical side of perithecial stroma. Perithecia, 250–390 × 110–130 μm, obovate, periphysate, with a dark brown ostiolar region. Asci 140–220 × 2.5–5.6 μm, cylindrical, containing 8 ascospores with a hemispherical refractive tip. Ascospores 130–210 × 1 μm, hyaline, filamentous, with several indistinct septa. Conidial state (ephelidal),
36–72 × 1.2–1.6 μm, holoblastic, hyaline, narrowly cylindrical to filiform, with 1–3 septa (Sullivan et al., 2001).

Other species in genus: None.

Sullivan et al. (2001) hypothesized that *Neoclaviceps monostipa* is an evolutionary intermediate of genera *Balansia* and *Claviceps*. Morphologically, the conidial state resembles that of the genus *Balansia*. *Neoclaviceps* resembles *Claviceps* in its infection of individual florets and its production of stipitate stromata. Recently, John Walker has indicated that the Australian *Claviceps phalaridis* possesses a conidial state that is similar to that of *N. monostipa* (John Walker, personal communication, 2001).

### 3.16. Genus: *Neocordyceps* Kobayasi

*Type species*: *Neocordyceps kohyasanensis* Kobayasi.

*Habit and type host*: Stromata on fruit of *Tripterospermum japonicum*.

*Collected from*: Mt. Kohya, Japan, Asia.

*Description of type species*: Stromata approximately 20 mm in length, clavate. Stipe 19 × 0.4–0.5 mm, slender, smooth, gray to white, surrounded by hyphae. Ascogenous portion, elongate, 9 × 1 mm.
Perithecia 43–45 × 19–20 μm, immersed, pyriform to ovoid, purple to black, with emergent ostiole. Asci 60 × 3.5 μm, with a thickened cap 1.5 μm in diameter. Ascospores 30–32 × 1.5–2 μm, elongate fusiform, 8–10 septate (Kobayasi, 1984).

Other species in genus: None.

3.17. Genus: Neomunkia Petrak

Type species: Neomunkia sydowii Petrak.
Habit and type host: Stromata on culms of Chusquea sp.
Collected from: Province of Tungurahua, Ecuador, South America.
Description of type species: Stroma 5–12 mm in diameter surrounding culm, globose to subglobose, solitary, punctate, gray to black, brittle when dry, gelatinous to fleshy when wet, minutely verrucose, thick epidermis (1–1.5 mm), plicate lengthwise in some areas, whitish in cross section. Epidermis made of layers, each measuring 8–20 μm in width. Conidia 2.5–3.5 μm, globose to subglobose, hyaline to amber (in masses), produced pleurogenously from conidiophores inside a continuous, irregular, acervulus-like cavity (50–80 μm in depth) covering a large portion of the stroma surface (Petrak, 1947).
Some additional taxa: None.

3.18. Genus: Parepichloë Reddy & White

Type species: Parepichloë cinerea (Berk. & Br.) White & Reddy = Epichloë cinerea Berk. & Br. = Epichloë sporoboli (Berk. & Br.) Teng.
Habit and type host: Stromata on inflorescences of Sporobolus indicus.
Collected from: India and China, Asia.
Other species in the genus: P. bambusae, P. cynodontis, P. oplismeni, P. sasae, P. sclerotica, and P. volkensii.

Parepichloë is distinguished from Epichloë in that: (1) Parepichloë occurs on warm-season grasses and Epichloë occurs on cool-season grasses; (2) the anamorph Neotyphodium is associated with Epichloë, while no anamorph has been connected to Parepichloë.

*Type species:* Phytocordyceps nunchukispora Su & Wang.

*Habit and type host:* Stromata on fruit of Beilschidia erythrophloia.

*Collected from:* National Taiwan University Experimental Forest, Taiwan, Asia.

*Description of type species:* Stroma 13.8–22.4 × 0.3–0.9 mm, clavate; stipe, 6.8–8.4 × 0.2–0.5, yellow–orange, cylindrical. Perithecial stroma 5.7–14.2 × 0.8–0.9 mm, yellow, clavate, with a long narrow longitudinal furrow (free of perithecia), 3.2–10.1 × 0.1–0.2 mm. Perithecia, 95–145 × 50–60 μm, pyriform, nearly superficial. Ascii, 75–105 × 2.1–3.1 μm, unitunicate, cylindrical with an enlarged apex penetrated with a fine pore. Ascospores, 90–110 × 1.2 μm, hyaline, 3–4 septate; fusiform, terminal, 20–30 × 1.2 μm, united into pairs by a filiform extension, 60–70 × 0.1 μm; does not form part spores. Conidial state, phialidic, 30–50 × 2.0–3.0 μm, simple to highly branched, developing from ascospores. Conidia 2.5–10 × 1.5–3.0 μm, hyaline, ellipsoid-cylindrical, smooth walled, 0–1 septate (Wang, 1986; Eriksson and Hawksworth, 1986).

*Other species in genus:* None.

Eriksson and Hawksworth (1986) placed *P. nunchukispora* in *Cordyceps* subg. *Bolacordyceps* based on the similarity of the interconnected terminal fusiform ascospores. However, there are differences between these species. The perithecial size of *C. nunchukispora* is less than half that of *C. bifusispora*. *Cordyceps ninchukispora* appears to be a plant pathogen, while *C. bifusisora* is an insect pathogen (Eriksson, 1982). Differences are also evident in the characters of the perithecial stroma. *Cordyceps ninchukispora* has perithecia that are nearly superficial, while *C. bifusisora* has immersed perithecia with only the ostiole exposed from the stromal surface. A further evaluation is needed to determine whether these two species should be included with the same genus.

3.20. Genus: Shimizuomyces Kobayasi

*Type species:* Shimizuomyces paradoxus Kobayasi.

*Habit and type host:* Stromata on fruit of Smilax sieboldi.

*Collected from:* Ueno-mura, Tano-gun, Gumma, Japan, Asia.

*Description of type species:* Mycelium on host, white, 2.5–3 μm in diameter, reticulate to pulvinate. Stroma erumpent from host fruit, 15–40 mm tall, clavate to cylindrical, fleshy, singular, rarely in aggregates of 2–7. Stipe, 10–30 × 0.5–1.2 mm, cylindrical, white to pallid yellow, smooth, composed of hyaline mycelium (3 μm in diameter). Perithecial
stroma 5–15 × 1–2 mm, cylindrical to clavate, differentiation between stipe and origin of perithecial stroma is inconspicuous, white to pallid yellow, papillate; peridium 25–30 μm thick, covered with reticulated, septate mycelium. Perithecia 350–400 × 200–250 μm, 2/3 immersed, pyriform to ovoid. Asci, 100–130 × 6–7 μm falcate to cylindrical, 2–6 ascospores per ascus, with a thickened apex. Ascospores 60–75 × 2–2.5 μm, fusiform to falcate, with 3–7 septa. After ejecting from the ascus, ascospores deform, 1–2 internal cells swell to attain 6–7 μm in diameter, other cells may curl and wrinkle (Kobayasi, 1981).

Other species in genus: S. kibianus Kobayasi & Shimizu.


Type species: Stereocrea schizostachyi Sydow & Sydow.

Habit and type host: Culm of Schizostachyum sp.

Collected from: Luzon, Philippines, Asia.

Description of type species: Stroma, stipitate, singular, 5–8 mm tall, with multiple stipes aggregated and olivaceous to fuliginous toward base. Perithecial stroma 1.5–4.0 cm in diameter, covered with scurf, capitate, scutiform to subglobose, 2–5 mm in diameter (Kretzschmaria-like). Multiple perithecialstromata converge at apex and give the appearance of one large tuber. Perithecia, 400–480 × 150–180, elongate-pyriform, immersed. Perithecia crowded, appearing as darkly pigmented dots across perithecial stroma. Asci 100–145 × 7–15 μm, unitunicate, cylindrical, later becoming elongate-clavate, with expanded apex, containing 8 ascospores, aparaphysate. Ascospores 49–73 × 6.5–8.5 μm, elongate-clavate, yellow to green, with 7–15 septa, slightly roughened, long attenuating at base (Sydow and Sydow, 1917; Rossman et al., 1999).

Other species in genus: S. coccophila.


Type species: Ustilaginoidea virens (Cooke) Takah. = Ustilago virens Cooke = Sphacelothea virens (Cooke) Omori = Tilletia oryzae (Cooke) Pat. = Ustilaginoidea oryzae (Cooke) Brefeld = Tilletia oryzae (Cooke) Pat.

Habit and type host: Infects the inflorescence of Oryza sativa.

Collected from: Tinnevelly, India, Asia.

Figure: 10.
Description of type species: Pseudo-sclerotia 5–10 mm in diameter, first yellow–orange then green–black, subglobose with flattened sides; enclosing flower parts, forming between glumes; in cross section pseudo-sclerotia are white at center surrounded by concentric layers of yellow, orange, and green to black in rind. Primary conidia are 4–6 μm in diameter at maturity, spherical to elliptical, olive green, echinulate, produced pleurogenously on sterigmata-like bracts, covering pseudo-sclerotia. Secondary conidia, less than 1 μm in diameter, ovoid, produced at apex of germ tube from primary conidia (Ou, 1972; Singh and Dube, 1976; Rathaiah and Bhattacharya, 1993).


The teleomorphic state of *Ustilaginoidea virens* has been described as *Claviceps oryzae-sativae* Hashioka (1951) based on infections of individual florets and formation of sclerotial-like structures. However, phylogenetic work currently being conducted does not place *U. virens* in *Claviceps* or other existing teleomorphic genera of the Clavicipitaceae (Chapter 5, Fig. 5, this volume). Occurrence of the unique ustilaginoid conidial state and phylogenetic analysis support classification of *Ustilaginoidea* spp. apart from other existing genera, with some affinity to *Dussiella*.

4. HOST SUBSTRATE QUESTIONED

Often, insect pathogenic fungi are mistaken for plant pathogens. This is especially true for many scale insect pathogens, due to the lack of insect remnants after it has been consumed. This has been true for many *Hypocrella* spp., including the formation of the genus *Fleisheria* Penzig & Sacc., which was later found to be an insect pathogen and combined with *Hypocrella*. Some genera that
should be scrutinized further are Ascopolyporus, Cavimalum, Dussiella, Mycomalus, and Stereocrea, due to the similarities in the point of attachment with Hypocreella and the superficial hypothallus that forms along the phytoplane. Along with Hyperdermium, these genera may first parasitize the scale insect and then utilize the sugars released through the stylet for additional nutrients.

5. EXCLUDED GENERA

Aciculosporium Miyake (type species: A. take Miyake) has been referred to genus Balansia Speg. (Kao and Leu, 1976; Mohanan, 1998). This species is the causal agent of “witches’ broom of bamboo” in Asia. We therefore do not recognize genus Aciculosporium. Diehl (1950) considered Balansiella Hennings to be synonymous with Claviceps Tulasne. White et al. (1997) considered Balansiopsis Höhn to be synonymous with Balansia Spegazzini. The genus Acrospermum Tode has been suggested to belong to the Clavicipitaceae (Müller and von Arx, 1973; Sherwood, 1977), but was more recently determined to be a member of Acrospermataceae, Xylariales by Barr (1990).

Similarly, Oomyces, long considered to be clavicipitalean (Diehl, 1950), was found to possess bitunicate asci (Eriksson, 1981), and was placed in the family Acrospermataceae. Coscinaria Ellis & Everhart is a synonym for Oomyces (Rogerson, 1970). Globulina Spegazzini was considered to be clavicipitalean by Diehl (1950), but was suggested to belong in the Dothideales by Rossman (1987). Linearistroma linearis Rehm., the type of that genus, was transferred into Myriogenospora Atk., by White and Glenn (1994). Many early graminicolous clavicipitaleans were placed in genus Ophiodothis Saccardo. However, Diehl (1950) considered Ophiodothis to be a nomen confusum.

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1. INTRODUCTION

Members of the clavicipitalean tribe Balansieae are biotrophs and cause systemic infections of grasses or sedges. In some species mycelia are endophytic, and in others they are epibiotic. Some Balansieae have been found to have some positive effects on grass hosts, including deterrence of insect and/or mammal herbivory and increased drought tolerance (Clay, 1988).

Spegazzini (1885) differentiated the genus Balansia Speg. from Claviceps Tul. because the stromata covered the entire inflorescence. Diehl (1950), who treated exclusively American species, wrote the most recent treatment of Balansia and its allies over a half-century ago. Diehl distinguished genera based on presence or absence of the two types of conidia, including macroconidia or ephelidial conidia (Ephelis Fr.) and microconidia or neotyphodial conidia (Neotyphodium Glenn, Bacon & Hanlin). Species that produced both micro- and macroconidia were classified in Atkinsonella Diehl; those producing only macroconidia were classified in Balansia; while species believed to lack either
conidial state were classified in genus *Balansiopsis* Höhn. Luttrell and Bacon (1977) placed *Myriogenospora* Atk. in the tribe Balansieae based on its production of macroconidia and similar growth habit.

Diehl’s system of dividing species into genera based on presence or absence of conidial states has not proven to result in meaningful genera in all cases. Problems first became apparent when it was discovered that some species (e.g., *Balansiopsis pillulaeformis* and *B. gaduae*), which were believed to lack conidia, produce them under certain circumstances (Rykard et al., 1982; White et al., 1997). These findings made it clear that *Balansiopsis* could not be separated from *Balansia* on the basis of absence of conidial states. Further, studies on phylogenetic relationships among the Balansieae using ribosomal DNA sequence data failed to support the separation of *Atkinsonella* from *Balansia* (Kulda et al., 1997; Reddy et al., 1998a, b). It is clear that the system for recognizing genera and species within the Balansieae must include consideration of all available features of the species.

### 2. FEATURES OF THE BALANSIEAE

#### 2.1. Growth Habit

Many species of Balansieae endemic to the Americas are endophytic, including *Balansia aristidae*, *B. brunnans*, *B. claviceps*, *B. discoidea*, *B. gaduae*, *B. henningsiana*, *B. nigricans*, *B. obtecta*, and *B. strangulans*. In many of these species endophytic mycelium is located intercellularly in ground tissues of the stem axis and leaves. It may also be evident in vascular tissues in the immediate proximity of stromata. Endophytic mycelium is typically not or sparsely branched, septate, adherent to outer walls of parenchyma cells, and oriented with the long axis of leaves or culms. Endophytic mycelium may be assessed through microscopic examination of tissues scraped from culms or leaf sheaths, depending on the species.

Indeed, the majority of species on a worldwide basis are epibiotic (Leuchtmann and Clay, 1988; White, 1994). In these species all the mycelium is evident outside the host epidermis. All Asian and African Balansieae examined to date, including, for example, *Balansia andropogonis* and *B. asperata*, are epibiotic. In addition, several American species, including *Balansia clavula*, *B. cyperi*, *B. cyperacearum*, *B. hypoxylon*, *B. pillulaeformis*, *B. texensis*, and *Myriogenospora* spp., are epibiotic. In epibiotic species mycelium does not appear to grow endophytically to any extent in hosts.

#### 2.2. Stroma Location

The location on the host on which stromata develop is a stable characteristic for most species of Balansieae. Species forming stromata on inflorescence primordia
include *B. asperata*, *B. andropogonis*, *B. asclerotica*, *B. claviceps*, *B. clavula*, *B. cypri*, *B. hypoxylon*, and *B. obtecta*. Typically, mycelium of these species grows on the inflorescence initials, altering their structure by preventing development of epidermal and cuticular barrier layers. The absence of these barrier layers permits the flow of nutrients to the stroma, which ultimately aids in its development and hinders that of the grass inflorescence. Species forming stromata on grass culms at nodes include *B. ambiens*, *B. aristidae*, *B. brunnans*, *B. discoidea*, *B. gadiuae*, *B. nigricans*, and *B. strangulans* (Fig. 1). These species are nourished by mycelium that penetrates into vascular tissues; thus nutrients are believed to flow directly from phloem and xylem into the stromal mycelium. *Balansia epichloe* forms stromata on upper surfaces of leaves. *Balansia henningsiana* forms stromata on lower surfaces of leaves. In *B. henningsiana* and *B. epichloe*, endophytic mycelium penetrates into the vascular tissues of the host as in species with stromata at nodes.

Endophytic species of *Balansia* tend to form stromata on culms at nodes but may also form them on inflorescences and leaves. Epibiotic species usually form stromata on inflorescences, but some form them on leaves or other organs. *Myriogenospora atramentosa* and *M. linearis* produce stromata on folded or rolled leaves (Fig. 2). The ability of these species to alter the surface of host leaves by destroying the cuticle layer may permit the fungus to extract the

*FIGURE 1*  Stroma of *Balansia ambiens* on culm of host, bar = 5 mm.
nutrients it needs to nourish the epibiotic mycelium of the stroma. *Balansia cyperacearum* is unusual in that its stromata may form anywhere on the surface of its sedge hosts.

### 2.3. Perithecial Stroma Morphology

The morphology of the perithecial stroma is another feature that is useful for identification and classification of Balansieae. Many species form flat, spreading perithecial stromata, including *Balansia aristidae*, *B. brunnans*, *B. epichloë*, *B. henningsiana*, *B. gaduae*, *B. nigricans*, and *B. strangulans*. Diehl (1950) classified these species in his subgenus *Dothichloë* (Atk.) Diehl. Several species, including *B. andropogonis*, *B. hypoxylon*, *B. discoidea*, *B. pillulaeformis*,
and *B. texensis*, possess restricted, sessile, flat to pulvinate perithecial stromata (Fig. 3). These perithecial stromata develop in discrete spots on the stroma and generally do not fuse or otherwise become confluent. Several Balansieae, including *B. asclerotiaca*, *B. asperata*, *B. claviceps*, *B. clavula*, and *B. obtecta*, possess perithecial stromata that are stipitate (Fig. 4). These species generally have a well-differentiated stipe that elevates a globose stroma containing perithecia several millimeters above a mycelial subiculum.

### 2.4. Anamorphic Structures

Hyaline conidiogenous cells (*Acremonium*-like or *Neotyphodium*-like) bearing microconidia are formed in a felty layer on the stroma surface in a few species, including *B. andropogonis*, *B. hypoxylon*, and *B. texensis*. In the closely related genus *Epichloë*, comparable microconidia function as spermata in a heterothallic
mating process. Macroconidia (classified in Ephelis) are produced in moist masses in conidiomata on the stromata. In B. aristidae and B. texensis the macroconidia emerge from depressed conidiomata on the surface of the stromata. In B. claviceps, B. hypoxylon, and B. obtecta, moist masses of macroconidia are produced on discoid to cupulate conidiomata. Moist masses of macroconidia are formed uniformly on the surface of stromata of B. nigricans; and in ridges on stromata of B. epichloë. Macroconidia have been shown to function as spermatia in a heterothallic mating process in B. epichloë.

2.5. Ascospore Disarticulation

The ascospores of the majority of the Balansiae are filamentous and multiseptate. There are three patterns of ascospore disarticulation evident among balansioid fungi. In species of Myriogenospora, ascospore filaments disarticulate very early in development to form numerous ellipsoidal initials that reinitiate growth to form acicular ascospore units. In Balansia obtecta and the majority of species placed in subgenus Dothichloë, ascospores are ejected entire as filamentous, multiseptate (7–14 septa) units but soon disarticulate at septa to form 1-septate, straight, cylindrical units. In B. andropogonis, B. asperata, B. hypoxylon, and B. texensis, filaments disarticulate after ejection to form 1-septate part-ascospores that are curved and typically narrower on one end than the other.

2.6. Mating System

The mating systems in the Balansieae are not well understood. Several studies have suggested that heterothallism with two mating types is the more common mating system in the Balansieae (Morgan-Jones and White, 1989; White et al.,
1995; Leuchtmann and Clay, 1989). In *Balansia obtecta*, sclerotia are formed on inflorescences of *Cenchrus echinata* or other hosts. Sclerotia over-winter and in the spring germinate to produce cupulate conidiomata bearing masses of spermatia (ephecidial conidia). Mixed in among the conidiomata are clusters of gold to tan, multisepate, receptive hyphae. Crossing experiments have demonstrated that perithecial stromata will develop from beneath the tufts of receptive hyphae only after spermatia of a compatible mating type are deposited on the receptive hyphae. Similar crossing experiments were conducted using stromata of *Balansia epichloë*. Insects may be involved in vectoring spermatia between compatible stromata of *Balansia*. In the closely related clavicipitalean group *Epichloë*, flies of genus *Botanophila* have been shown to mediate a fertilization process that has been compared to pollination of Angiosperms (Bultman et al., 1995). Grasshoppers have been observed to feed on conidia of stromata of *Balansia henningsiana*, however, effective vectoring of spermatia has not been demonstrated (White, unpublished).

It is possible that *Myriogenospora* possesses a homothallic mating system because perithecia develop on stromata very rapidly and uniformly without a period in which stromata may be fertilized. Further, the developing conidial stromata of *Myriogenospora* are fully surrounded by leaves prior to perithecial development. This would seem to exclude fertilization by insects. However, it is also possible that *Myriogenospora* possesses pseudo-homothallism. The acicular ascospores of at least one species, *M. atramentosa*, are ejected *en masse* from asci and would be expected to contain a mixture of the genotypes resulting from meiosis. It is possible that fusion of mating types, if they exist in this species, may occur between ascospores or the *Ephelis* conidia that result from the germinating ascospores on the surfaces of plants onto which the masses of ascospores are ejected. Thus plant infections are set up as small populations of genotypes. This mating system would permit *Myriogenospora* to produce perithecia and ascospores rapidly and consistently without the need for insect vectors of spermatia. It also would permit outcrossing in cases where a plant becomes infected by ascospore masses from multiple genotypes of maternal individuals. The precise mating system of *Myriogenospora* and many other Balansieae awaits experimental elucidation.

3. HOST RELATIONS

3.1. Biotrophic Nature

The Balansieae consists of biotrophic symbionts that infect both grasses and sedges. They complete their entire life cycle on or in host tissues. Nutrients are extracted from the plant to sustain mycelial growth and form reproductive structures. It is believed that these fungi infect their hosts through seeds or
seedling plants (Hill, 1994). Precisely how Balansieae set up primary infections of hosts is still uncertain; however, they eventually produce stromata on plants. Typically stromata consist of a combination of living plant tissues and mycelium. Plant tissues embedded in stromata remain alive but may be modified structurally.

3.2. Host Modifications

*Balansia* and *Myriogenospora* form their reproductive structures on rapidly developing inflorescences and leaves of their hosts (White et al., 1997). A study on the enzymatic capabilities of the Clavicipitaceae revealed that the Balansieae lack cellulases (Lewis et al., unpublished). However, they were found to produce lipase, and proteinase. These enzymes may be involved in cell wall modification. Lipases may be used to degrade the waxy cuticle of the plant. The waxy cuticle reduces evaporation from the surface of the grass host. Without the cuticle, nutrients may flow from the plant into the fungal stroma. Epidermal cells associated with the stroma of *Myriogenospora atramentosa* and *B. epichloë* are typically hypertrophied and show disruptions of the structure of the cuticle (Rykard et al., 1985; White and Glenn, 1994). Additionally, symptoms such as witch’s brooms (*B. gaduae*), slight dwarfing of culms (*B. henningsiana*), as well as deformation of the inflorescence and flag leaf (*B. hypoxylon*, *B. texensis*, *B. pillulaeformis*) has been proposed to be due to the production of auxin-like alkaloids (Hill, 1994).

3.3. Nutrient Specialization

White et al. (1991) performed substrate utilization studies in *Balansia hypoxylon*, *B. texensis*, and *B. epichloë*. These studies showed that most members of the Balansieae utilize several noncarbohydrate energy sources including oil and paraffin droplets. The data suggest that hydrolyzed hydrocarbon fragments and glycerol may diffuse through membranes of the fungi and be used as energy sources. The same study implied that sugar concentration may partly regulate mycelial development and stromata formation. Similar research has demonstrated that Balansieae produce extracellular invertases which cleave sucrose into the monomers fructose and glucose (Lam et al., 1994; Lewis, unpublished). The presence of extracellular invertases suggests that Balansieae do not absorb sucrose, but instead break it down to smaller molecules prior to absorption.

3.4. Benefits to the Host

In most plant–microorganism relationships, there are costs and few benefits for the host plants in the interaction. However, infections by *Balansia* species can yield benefits in addition to any adverse effects on hosts. For instance,
endophytic clavicipitaleans have been shown to increase drought tolerance to some hosts (Funk and White, 1997). It is believed that Balansieae in general have similar effects on their hosts, although this possibility has not been exhaustively tested.

Balansieae may be capable of increasing fungal disease resistance. Several endophytic relatives of Balansieae have been linked to the deterrence of fungal pathogens. Clarke et al. (unpublished) found a decline in occurrence of *Sclerotinia homeocarpa* (the causal agent of dollar spot) in fine fescue plants infected with *Epichloë festucae*. The resistance mechanism may be attributed to antifungal compounds produced by the endophyte. Moy et al. (2000) proposed that epiphyllous mycelial nets produced by *Neotyphodium* sp. play a role in decreasing fungal pathogens on the leaf surface. This niche exclusion is attributed to the “defensive net” of mycelium occupying spaces and excluding pathogens from the phylloplane. Thus, epibiotic Balansieae may utilize this strategy to protect their host plant from potential pathogens. In fact, *Balansa cyperi* has been found to increase resistance to *Alternaria* and *Rhizoctonia* species (Stovall and Clay, 1991).

In some cases, infection by Balansieae has been associated with deterrence to insect and mammalian herbivores (Clay et al., 1985; Hardy et al., 1986; Siegel et al., 1985). *Paspalum dilatatum* infected by *Myriogenospora atramentosa* and *Cyperus virens* infected by *Balansa cyperi* have been shown to cause reduced survival rates and weight gain in fall armyworm populations (Clay et al., 1985). Toxic alkaloids produced by the fungi are thought to be the primary cause of deterrence (Bacon et al., 1975). Experimental evidence has indicated that *B. claviceps*, *B. cyperi*, *B. epichloë*, *B. henningsiana*, *B. obtecta*, and *B. strangulans* produce a variety of ergot alkaloids (Bacon, 1985; Powell et al., 1990).

### 3.5. Toxic Syndromes

Two syndromes that stem from clavicipitalean grass infections include “fescue foot” and “ryegrass staggers,” which disable cattle grazing in infected pastures. *Balansa epichloë* infections in the grasses *Sporobulus poirettii*, *Eragrostis hirsuta*, and *Panicum anceps* as well as *B. henningsiana* in *E. hirsuta*, *P. anceps*, and *Andropogon* spp. were observed in toxic fescue pastures (Bacon et al., 1975). In addition to farm animal toxicity, farmers may have to deal with decreased seed set which is a product of the destruction of inflorescences due to the formation of the stroma. This symptom, which has classically been termed “choke,” is known to occur on *Andropogon*, *Cenchrus*, *Cynodon*, *Panicum*, *Setaria*, and *Sorghum* (Diehl, 1950). The decreased yield may be important to seed distributors of these forage grasses.
Likewise, choke disease has been documented on rice. In Asia choke disease on rice is termed “Udbatta” or “silver leaf disease”. This is named for the epibiotic mycelium covering the upper surface of the leaves. In Asian countries, Ephelis oryzae Syd. is the causal agent of silver leaf disease and decreases the production of rice grains (Govindu and Thirumalachar, 1960). It is not known whether alkaloid toxicity occurs on Ephelis-infected rice.

4. BALANSIEAE IN RELATION TO OTHER CLAVICIPITACEAE

A maximum likelihood tree using the 6ST-GTR + G + I evolutionary model was constructed using genbank sequences from several members of the Clavicipitaceae. According to the analysis (Fig. 5), Atkinsonella is placed into the genus Balansia (52% support). Myriogenospora was also grouped in Balansia; however, we propose that it should be retained as separate from Balansia due to the long branch length illustrated in the tree and by morphological differences such as ascus and ascospore size. In this treatment, we classify species of Atkinsonella in genus Balansia but retain Myriogenospora as a distinct genus of Balansieae.

In 1885 Spegazzini described the close relationship between Balansia and Claviceps Tul, but decided to separate the two genera. Our analysis supports Spegazzini’s decision, showing a clear separation of Claviceps from Balansia and Epichloë. Diehl (1950) placed Epichloë and Balansia in tribe Balansieae. This grouping is not supported in our analysis.

5. ENUMERATION OF THE SPECIES

5.1. Balansia ambiens (Moell.) Diehl (Diehl, 1950)

*Hosts:* Leersia grandiflora, Olyra sp.
*Stroma location:* Inflorescence primordium or culm.
*Perithecial stromatal form:* pulvinate.

5.2. Balansia andropogonis Syd. & Butl. (Saccardo, 1926)

*Hosts:* Centotheca latifolia, Centococcum oxyphyllum.
*Stroma location:* inflorescence.
*Perithecial stromatal form:* sellile, flat to pulvinate.

5.3. Balansia aristidae (Atk.) Diehl (Diehl, 1950)

*Host:* Aristida sp., Aristida purpurascens.
*Stroma location:* Below the node, covering part of the adaxial leaf surface.
*Perithecial stromatal form:* Flat, spreading.
FIGURE 5 A maximum-likelihood tree using the 6ST-GTR + G + I evolutionary model.
5.4. **Balansia asclerotiaca. P. Henn (Diehl, 1950)**

*Hosts:* Orthoclada laxa.
*Stroma location:* Inflorescence.
*Perithecial stromatal form:* Stipitate.

5.5. **Balansia asperata Sacc. (Saccardo, 1926)**

*Hosts:* Panicum carinatum, Panicum patens.
*Stroma location:* Inflorescence.
*Perithecial stromatal form:* Stipitate.

5.6. **Balansia brunnans (Lewis et al., 2002)**

*Host:* Panicum xalapéns Kunth.
*Stroma location:* On culms below nodes.
*Perithecial stroma form:* Flat, spreading.

5.7. **Balansia claviceps Speg (Diehl, 1950)**

*Host:* Setaria sp.
*Stroma location:* Inflorescence.
*Perithecial stromatal form:* Stipitate.

5.8. **Balansia clavula Berk. & Curt. (Saccardo, 1883)**

*Hosts:* Paspalum ciliatifolium, Paspalum debile, Paspalum pubescens.
*Stromata location:* Inflorescence primordia.
*Perithecial stromatal form:* Stipitate.

5.9. **Balansia cyperacearum (Berk & Curt.) Diehl (Diehl, 1950)**

*Hosts:* Cyperus spp., Scleria bracteata.
*Stroma location:* Abaxial surface of leaves or partially surrounding the culm at nodes.
*Perithecial stromatal form:* Effuse, flattened.

5.10. **Balansia cyperi (Edg.) Diehl (Diehl, 1950)**

*Hosts:* Cyperus sp., Cyperus virens.
*Stroma location:* Enclosing apex of culms, including the base of involucral leaves and inflorescence primordia.
*Perithecial stromatal form:* Spherical to subspherical.
5.11. *Balansia discoidea* (Beibl.) P. Henn. (Henn, 1904)

*Hosts:* Andropogon sp., Bouteloua curtipendula, Chloris distichophylla, Pennisetum purpureum, Trichloris crinita, Trichloris mendocina.

*Stroma location:* Encircling culm just below the node.

*Perithecial stromatal form:* Flat, sessile.

5.12. *Balansia epichloe* (Weese) (Diehl, 1950)

*Host:* Andropogon sp., Eragrostis refracta, Panicum sp., Sporobolus sp., Triodia flava.

*Stroma location:* Adaxial leaf surface.

*Perithecial stromatal form:* Flattened.


*Host:* Bambusa arundinacea, Chusquea uniflora, Guadua sp.

*Stroma location:* On one side of the culm at the nodes or on the internode immediately below the node.

*Perithecial stromatal form:* Irregular, sessile.


*Hosts:* Ichnanthus candidus, Lasiacis sp., Oplismenus hirtellus, Setaria paniculifera.

*Stroma location:* Partially or completely encircling culms at nodes.

*Perithecial stromatal form:* Rugulose.

5.15. *Balansia hemicrypta* Diehl (Diehl, 1950)

*Hosts:* Aristida sp.

*Stroma location:* Inside the lemma.

*Perithecial stromatal form:* Stipitate, reddish.

5.16. *Balansia henningsiana* (Moell.) Diehl (Diehl, 1950)

*Hosts:* Andropogon sp., Panicum sp.

*Stroma location:* Lower surface of unrolling leaves.

*Perithecial stromatal form:* Effuse and lageniform.

5.17. *Balansia hypoxylon* (Pk.) Atk (Diehl, 1950)

*Hosts:* Andropogon sp., Aristida sp., Panicum sp., Uniola sp.
*Stroma location:* Enveloping the lower portion of the leaf and the immature inflorescence.

*Perithecial stromatal form:* Pulvinate, sessile.

5.18. *Balansia obtecta* Diehl (White et al., 1995)

*Hosts:* Cenchrus echinatus.

*Stroma location:* On sclerotia which form on the apex of culms; on developing inflorescences and some surrounding leaves.

*Perithecial stromatal form:* Stipitiate.

5.19. *Balansia nigricans* Speg. (White et al., 1996)

*Hosts:* Axonopus sp.

*Stroma location:* Culms at nodes.

*Perithecial stromatal form:* Raised, sessile, with a reflective black surface.

5.20. *Balansia pallida* (Wint.) (Diehl, 1950)

*Hosts:* Luziola peruviana.

*Stroma location:* Within florets.

*Perithecial stromatal form:* Subspherical.

5.21. *Balansia pillulaeformis* (Berk. & Curt.) Diehl (Diehl, 1950)

*Hosts:* Chasmanthium pilulaeformis, Uniola sp.

*Stroma location:* On inflorescence primordia and surrounded by a folded leaf.

*Perithecial stromatal form:* Hemispherical, sessile, flat to pulvinate.

5.22. *Balansia strangulans* (Mont.) (Diehl, 1950)

*Hosts:* Panicum spp.

*Stroma location:* On culms just below or on the nodes.

*Perithecial stromatal form:* Flat, spreading.

5.23. *Balansia texensis* (Diehl) Leuchtman et Clay (Diehl, 1950)

*Host:* Stipa sp.

*Stroma location:* Inflorescence primordia.

*Perithecial stromatal form:* Sessile, pulvinate.

*Hosts:* Andropogon sp, Axonopus sp, Cymbopogon sp, Eremochloa sp., Panicum sp, Paspalum sp, Saccharum sp.

*Stroma location:* Surface of the pre-emergent folded leaf.

*Perithecial stromatal form:* Linear with one row of perithecia.


*Hosts:* Chusquea sp., Olyra micrantha, Pariana sp.

*Stroma location:* Rolled leaves, erupting through the epidermis.

*Perithecial stromatal form:* Linear, with two rows of perithecia.

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1. INTRODUCTION

Epichloë is one of seven genera recognized within the tribe Balansieae of the Clavicipitaceae, which represent exclusively systemic biotrophs of graminoid hosts (Diehl, 1950; White, 1994b; White and Reddy, 1998; White et al., 2000). Other members of the family are either not systemic and perennial (tribes Clavicipeae, Ustilaginoideae) or are pathogens of invertebrates or other fungi (tribe Cordycipeae). A key feature of Epichloë is the endophytic lifestyle, which includes intercellular colonization of host tissues and frequent seed transmission. Most species of other balansiean genera (e.g., Atkinsonella, Balansia, Myriogenospora, Parepichloë) live epibiotically on host meristems and surfaces of leaves or culms. Epichloë species form light-colored, external reproductive structures, the stromata, which envelope inflorescences and the upper leaf sheaths of flowering culms (choke disease). The anamorphic state which is formed on the surface of the young stromata is classified in Neotyphodium (Glenn et al., 1996). Its one-celled conidia, typically ovoid to navicular in shape, are borne from simple phialids.
Neotyphodium conidia may also be produced in culture and are the only spore type of the related asexual Neotyphodium ssp., which have lost the capacity to develop Epichloë stromata. Species of both endophyte groups are restricted to hosts of cool-season grasses (Pooideae), predominantly to those indigenous to the Northern hemisphere [but see also Miles et al. (1998) and Cabral et al. (1999)].

Over the past decade, detailed studies of morphology and biology of Epichloë fungi together with the application of modern molecular techniques by various researchers have profoundly affected the taxonomy of that genus and our view of its origin and evolution. This chapter gives an overview on the current state of taxonomy and discusses issues of evolution in some recently recognized species of Epichloë. The related Neotyphodium endophytes will not be considered here, and readers are referred to Chapter 9 in this volume. Another focus of this chapter is the association of Epichloë with symbiotic flies of genus Botanophila, which serve as vectors of spermatia for mating and which may have contributed to the diversity of the genus. I discuss results of ongoing research on the fly mutualism and the role of Botanophila for Epichloë evolution.

2. LIFE CYCLES

Symbioses of grasses with Epichloë endophytes, which are usually maintained throughout the life span of the host, are very intimate and involve coordinated life cycles of both fungus and host. During the vegetative phase of grass development Epichloë species cause no visible symptoms of infection, but grow intercellularly in stems and leaves by typically forming convoluted, sparsely branched hyphae parallel to the long axis of plant cells (Fig. 1).

**FIGURE 1** Alternative life cycles of an *Epichloë* species with balanced transmission modes. In the asexual cycle the fungus grows intercellularly (B) in leaf sheaths of seedlings (A), meristems of flowering tillers (C), and inflorescences (D) infecting ovaries (F) in all florets (E); it infects aleurone cells (H) of developing seeds (G) and later, upon germination the embryo, and is thus transmitted vertically through successive host generations. In the sexual cycle the fungus also grows asymptotically in leaf sheaths and meristems, but then forms external stromata with conidia (I) around most or all developing inflorescences, aborting them in the process (choke); heterothallic mating is mediated by a *Botanophila* fly (J) transferring conidia (spermatia) of opposite mating type (K), on which perithecia containing asci develop (L), and filamentous ascospores are ejected (M); ascospores germinating with iterative cycles of asexual sporulation (N) mediate horizontal (contagious) transmission through infections of host florets (O) and then seed (P); alternatively, vegetative tissues may be invaded, leading to infections of tiller buds. (From Leuchtmann and Schardl, 1998.)
Endophytic hyphae are most abundant in nodal or basal leaf meristems and in leaf sheaths, but are sparse in leaf blades, and absent in roots (Hinton and Bacon, 1985; White, 1993). They may be clonally propagated in vegetatively formed tillers or in stolones of the grass. At the time when grasses start to develop flowering stems, fungal hyphae proliferate on the surface of inflorescence primordia and between the young leaves surrounding them, and eventually form a dense mycelial mat. This process effectively prevents further development of inflorescence primordia into flowers, so that seed production is suppressed. The external mycelium gives rise to a white conidial stroma from which, as a dense layer, *Neotyphodium* conidia are produced (White et al., 1991). *Epichloë* species are heterothallic, requiring the transfer of conidia (which then function as spermatia) from one stroma to another of the opposite mating type for sexual reproduction (White and Bultman, 1987; Leuchtmann and Schardl, 1998). The role of the vector of spermatia is taken by *Botanophila* flies, which consume spermatia and transport them endochorously through their alimentary tract (Bultman and White, 1988; Bultman et al., 1995). Following mating, perithecia containing ascii and ascospores develop in a thick peripheral layer around the entire stroma, which then turns yellow to orange from carotenes formed in the perithecial wall. The filamentous, multisepitate ascospores, which are the propagules for horizontal transmission, are actively discharged from the ascii and become wind-dispersed (Raynal, 1991; Welch and Bultman, 1993). Contagious infections may occur either directly by ascospores or by infective conidia after iterative cycles of conidiation (Bacon and Hinton, 1991). Exact mechanisms of infections under natural conditions are not fully understood and may vary for different host species. One proposed and experimentally confirmed mechanism is the invasion of ovules and seed after infection of grass florets via the stigmata (Chung and Schardl, 1997). Alternative routes of infection include wounds of cut stubble (Western and Cavett, 1959), or colonization of vegetative tillers of grass hosts, presumably by invading the meristematic zone of tiller buds (Brem and Leuchtmann, 1999).

In the more benign asexual cycle, *Epichloë* endophytes (and all *Neotyphodium* species) remain asymptomatic (Fig. 1). As floral primordia are formed, endophytes grow into ovules, proliferate in the nucellus tissues, and later colonize the embryonic axis of the developing seed, which leads to vertical transmission (Freeman, 1904; Philipson and Christey, 1986). Hyphae in the remnant nucellar layer form a conspicuous mat between the aleuron and the seed coat (White et al., 1991). Symptomless endophytes may also invade stamen filaments and anther walls, but have never been found in pollen grains, and apparently are not disseminated via paternal structures (Sampson, 1933; Hinton and Bacon, 1985). Vertical transmission of endophytes in infected tillers occurs at nearly 100% efficiency (Siegel et al., 1984). However, seeds which are free of endophyte may occasionally be produced when shoot meristems of individual
tillers escape the infection (Kirby, 1961; Ravel et al., 1997). Furthermore, endophyte viability in the seeds is limited and declines rapidly under high relative humidity at room temperature (Welty et al., 1987). Thus, some of the seeds germinating in the season following their production may have lost the endophyte.

There is considerable variation in the life cycles of different *Epichloë* species and in different host populations (White, 1988; Leuchtmann and Clay, 1997). In some species, stromata are formed invariably on all tillers of infected grass hosts, so that seed production is completely suppressed. This type of symbiosis represents the antagonistic extreme. Other species display both sexual and asexual cycles (balanced transmission) on different tillers of an infected plant, or on different subsets of individuals of a host population where seed transmission is often predominant. These associations are considered to be more mutualistic. In a third category, no stromata are formed on any of the infected plants, and seed transmission is the only means of dispersal. The latter group includes genetically distinct strains derived from sexual *Epichloë* spp. and all *Neotyphodium* species.

### 3. TAXONOMY AND EVOLUTION

#### 3.1. Species Diversity and Mating Populations

Because of rather limited morphological variation, all sexual *Epichloë* fungi infecting cool-season grasses were originally placed in one collective species, *E. typhina*. Only recently, more detailed morphological studies combined with interfertility tests and molecular evidence has led to the formal description of a number of distinct species which are mostly host-specific and confined to one or few host grass genera of a single tribe (Table 1). The exception is *E. typhina sensu stricto*, which appears to have a broader host range but may include several incipient host-specific species, as will be discussed later. Some but not all species may be distinguishable based on morphological characteristics which include size or disarticulation patterns of filiform ascospores, and density of perithecia formed within the stromata. More important, each of the 10 currently recognized *Epichloë* species, with one exception, has been identified as a distinct mating population or biological species based on mating tests (Scharld et al., 1997). The exception is *E. clarkii*, which is interfertile with *E. typhina* experimentally, but for reasons not fully understood appears to be reproductively isolated in nature. This morphospecies infects only *Holcus lanatus* and is characterized by a unique ascospore morphology. Biological species of *Epichloë* differ in their host range with no species overlap and are restricted to either Eurasia or North America following the native distribution of the host grasses. Finally, the life cycle is often distinctive for a species but may also be variable among strains of a species.
<table>
<thead>
<tr>
<th>Species</th>
<th>Host genera</th>
<th>Native distribution</th>
<th>Life cycle</th>
<th>Morphological characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. amarillans</em></td>
<td>Agrostis, Calamagrostis, Sphenopholis</td>
<td>NA</td>
<td>B</td>
<td>Ascospores not disarticulating, 217 ± 5 × 1.0 μm; perithecial density particularly low</td>
<td>(White, 1994a, b; Schardl and Leuchtmann, 1999)</td>
</tr>
<tr>
<td><em>E. baconii</em></td>
<td>Agrostis, Calamagrostis</td>
<td>E</td>
<td>S</td>
<td>Ascospores disarticulating, part-spores cylindrical, 1-septate, 24 ± 5 × 1.8 ± 0.2 μm</td>
<td>(White, 1993; Leuchtmann, 1997)</td>
</tr>
<tr>
<td><em>E. brachyelytri</em></td>
<td>Brachyelytrum</td>
<td>NA</td>
<td>B</td>
<td>Ascospores not disarticulating, 290 ± 41 × 1.2–1.4 μm; asci with flattened cap</td>
<td>(Schardl and Leuchtmann, 1999)</td>
</tr>
<tr>
<td><em>E. bromicola</em></td>
<td>Bromus, Hordelymus</td>
<td>E</td>
<td>S/A</td>
<td>Ascospores not disarticulating, 267 ± 33 × 1.7 ± 0.3 μm</td>
<td>(Leuchtmann and Schardl, 1998)</td>
</tr>
<tr>
<td><em>E. clarkii</em></td>
<td>Holcus</td>
<td>E</td>
<td>S</td>
<td>Ascospores disarticulating, part-spores spear shaped, multisepitate, 46 ± 16 × 2.3 ± 0.3 μm</td>
<td>(White, 1993)</td>
</tr>
<tr>
<td><em>E. elymi</em></td>
<td>Elymus</td>
<td>NA</td>
<td>B</td>
<td>Ascospores not disarticulating, 423 ± 63 × 1.2 ± 2.0 μm</td>
<td>(Schardl and Leuchtmann, 1999)</td>
</tr>
<tr>
<td><strong>E. festucae</strong></td>
<td><em>Festuca, Koeleria</em></td>
<td>E</td>
<td>P</td>
<td>Ascospores 250–390 × 1.6–2.0 μm, disarticulating in the middle</td>
<td>(Leuchtmann et al., 1994; Craven et al., 2001)</td>
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<td>Leuchtmann,</td>
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<td>Schardl &amp; Siegel</td>
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<tr>
<td><strong>E. glyceriae</strong></td>
<td><em>Glyceria</em></td>
<td>NA</td>
<td>S</td>
<td>Ascospores not disarticulating, 366 ± 37 × 1.2−1.6 μm;</td>
<td>(Schardl and Leuchtmann, 1999)</td>
</tr>
<tr>
<td>Schardl &amp;</td>
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<td></td>
<td>perithecial density low</td>
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<td>Leuchtmann</td>
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<tr>
<td><strong>E. sylvatica</strong></td>
<td><em>Brachypodium</em></td>
<td>E</td>
<td>S/B</td>
<td>Ascospores not disarticulating, 221 ± 50 × 1.6 ± 0.1 μm</td>
<td>(Leuchtmann and Schardl, 1998)</td>
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<td>Leuchtmann</td>
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<td>&amp; Schardl</td>
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<tr>
<td><strong>E. typhina</strong></td>
<td>(Pers.: Fr.) Tul.</td>
<td>E</td>
<td>S/B</td>
<td>Ascospores not disarticulating, 176 ± 34 × 1.6 ± 0.2 μm</td>
<td>(White, 1993; Leuchtmann, 1997; Craven et al., 2001)</td>
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<td>(Pers.: Fr.)</td>
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<td>Tul.</td>
<td>Anthoxanthum,</td>
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<td>Arrhenatherum,</td>
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<td>*Brachypodium,</td>
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<td>*Dactylis, Lolium,</td>
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<td></td>
<td>*Phleum, Poa,</td>
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<td></td>
<td><em>Puccinellia</em>^c</td>
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</table>

^a E = Eurasia; NA = North America.  
^b S = sexual only; A = asexual; B = balanced (sexual and seed-transmitted).  
^c Leuchtmann, unpublished data.
infecting different hosts. For example, *E. bromicola* is strictly sexual on *Bromus erectus*, but asexual and seed-transmitted on *B. ramosus* and *B. benekenii* (Leuchtmann and Schardl, 1998). In addition, an asexual form of *E. festucae* infecting *L. perenne*, on which stromata are not expressed but which appeared to be sexually competent in matings with *E. festucae* stromata, was recently found (Moon et al., 2000; Chapter 9, this volume).

### 3.2. Phylogenetic Relationships

Although taxonomy and phylogenetic relationships among most higher taxonomic categories of the Clavicipitaceae are not well developed, it is now firmly established based on partial sequences of 26S nuclear rDNA that *Epichloë* (together with their anamorphic relatives) represent a monophyletic genus which is closely related to other plant-associated genera of the family but more distantly related to insect-pathogenic *Cordyceps* species (Kuldau et al., 1997). Discordant elements of *Epichloë*, such as *E. cinerea* and *E. bambusae*, formerly described from various hosts from the African and Asian tropics, have been excluded and are mostly transferred to *Balansia* or the new genus *Parepichloë* (White and Reddy, 1998). Early phylogenetic analysis of the sexual species of *Epichloë* relied mostly on the noncoding regions of the β-tublin gene (*tub2*), in which most but not all biological species corresponded to well-defined clades associated with particular host species, genera, or tribes (Schardl et al., 1997; Schardl and Leuchtmann, 1999). The exception was *E. sylvatica*, which was placed within the paraphyletic *E. typhina* complex (Fig. 2). Here, it is obvious that the classification scheme based on interfertility groups does not correspond with a phylogenetic species concept. An argument which may explain the apparent discrepancy of species concepts is that single-gene phylogenies might not reflect the true evolutionary relationships of isolates. Similarities between sequences can arise from mechanisms other than common ancestry, for example, through convergence or gene conversion.

The most comprehensive phylogenetic study of *Epichloë* adopted a multiple-gene approach to measure the accuracy of gene trees by including sequences of two additional nuclear genes, translation elongation factor 1-α (*tef1*) and actin (*act1*) (Craven et al., 2001). Maximum parsimony (MP) analysis of the combined data set of *act1*, *tub2*, and *tef1* sequences essentially gave the same tree topology as with *tub2* alone (Fig. 3). At the root, with 100% bootstrap support, species of *Epichloë* were divided into two major groups, a main group including seven species with mostly balanced transmission strategies, and the *E. typhina* complex with predominately sexual, horizontally transmitted endophytes including the wide-host-range species *E. typhina*, and *E. clarkii* and *E. sylvatica*. A very strongly supported clade within the *E. typhina* complex, referred to as crown clade, included *E. clarkii* and most isolates of *E. typhina*, while *E. sylvatica* and *E. typhina* isolates from two *Poa* hosts were arranged basal...
to that clade. In single-gene phylogenies all major clades that were strongly supported by the combined data set were also strongly supported by *tef1* and *act1* phylogenies, whereas several less supported clades remained unresolved or were incongruent, notably the position of *E. glyceriae* and *E. brachyelytri* in the *tef1* trees, and the placement of the isolates within the basal clades of the *E. typhina* complex in the *act1* trees (Craven et al., 2001). Incongruence between gene trees in placement of taxa is expected if clade members are potentially interbreeding, as would be the case in the *E. typhina* complex. Results of the multigene phylogenies further indicate that different evolutionary patterns may be associated with different transmission modes to new host plants. A mostly

**FIGURE 2**  *tub2* gene tree of *Epichloë* spp. form different hosts based on maximum parsimony analysis. The bar represents 5 inferred nucleotide substitutions. Numbers at branches are the percentages of trees containing the corresponding clade based on 500 bootstrap replications. Values greater than 70% are considered supportive of the clade. (Modified from Schardl and Leuchtmann, 1999.)
FIGURE 3  Phylogenetic relationships of representative isolates of all known *Epichloë* species and the outgroup *Claviceps purpurea* based on the combined data set of *act1*, *tub2*, and *tef1* intron sequences. Hosts of *Epichloë* species
cladistic pattern of speciation in which phylogenetic and biological species concepts correspond is evident in endophyte lineages with balanced vertical and horizontal transmission, whereas cladistic evolution is less evident in strictly horizontally transmitted and therefore less host-specific species. In explaining such outcome, Craven et al. (2001) speculate that dependence on horizontal transmission may counteract host specialization and delay speciation that cause conflict between the biological and phylogenetic species concepts as exemplified in *E. typhina* and *E. sylvatica*.

### 3.3. Evolution of Reproductive Strategies

Since *Epichloë* endophytes differ in their life-cycle patterns, particularly with regard to the mode of transmission, the question may be asked which pattern represents the ancestral state. Schardl and Wilkinson (2000) have argued that the balanced symbiosis in which both transmission modes occur may be the most ancestral, because loss of a trait is more likely than a gain. Purely horizontally transmitted and purely vertically transmitted associations each involve a loss of one form of endophyte transmission. In fact, established phylogenies indicate that in the major clades of the genus, endophyte lineages with a balanced life cycle are basal (e.g., *E. brachyelytri* and *E. typhina* on *Poa nemoralis*), while purely horizontally transmitted and purely seed-transmitted lineages tend to be derived (Figs. 2 and 3).

An alternative view held by this author is that ancestral *Epichloë* parasites may have been non-seed-transmitted and perhaps epibiotic, because emergence of this type of association would have required much less coordinative efforts by both partners. Among species of the other genera of the tribe Balansieae (*Atkinsonella, Balansia, Echinodothis, Myriogenospora*, and *Parepichloë*), epibiotic associations are dominating and vertical transmission appears to be exceptional, documented only for the genus *Atkinsonella* (White, 1994b).

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Unfortunately, based on available molecular evidence, it cannot be determined which of the genera are closest to *Epichloë* and thus might share common ancestors (Kuldau et al., 1997; White and Reddy, 1998). The various clades representing different genera seem to have emerged nearly simultaneously. It may be speculated that endophytism and the development of seed transmission in *Epichloë* were two independent evolutionary steps which occurred successively during emergence of balanced associations. Such an assumed ancient, purely horizontally transmitted lineage could be *E. bromicola* on *B. erectus*, which is placed most basal in the *tub2* phylogeny of European species (Leuchtmann and Schardl, 1998). However, when European and North American species are combined in the analysis, the position of *E. bromicola* in the tree appears to be more derived (Fig. 3). Therefore, it remains unclear which type of association may represent the ancestral state and whether ancient lineages still exist among the *Epichloë* species currently known. Clearly, in the course of radiation of the genus, evolution has moved in both directions several times. The purely horizontally transmitted *E. typhina* appears to be ancestral to *E. sylvatica*, which in turn gave rise to the purely asexual subpopulation on *B. sylvaticum*. Similarly, *E. bromicola* on *B. erectus* is ancestral to purely seed-transmitted strains on two other *Bromus* hosts. Both cases will be discussed in more detail later. The reverse may have occurred in the evolution of *E. baconii* and *E. glyceriae*, which both have ancestors with balanced transmission modes (Fig. 2).

### 3.4. Host-Related Genetic Diversity

The biological species of *Epichloë* defined as experimental mating populations (Leuchtmann et al., 1994; Schardl et al., 1997) are often genetically diverse and may be more comprehensive than actual mating populations in nature. The degree of such diversity may be best illustrated by allozyme data, which provide a conservative estimate of genetic divergence of endophyte populations (Murphy et al., 1996) and which are depicted for representative host strains of the European species in Fig. 4 (Leuchtmann and Schardl, 1998). Most within-species divergence appears to be host-related, and multiple isolates of the same host are usually very similar (the exceptions are some isolates of *E. typhina* from *D. glomerata*, and *E. sylvatica* from *Bp. sylvaticum*). The largest distances between host strains of the same species are found within *E. typhina* (e.g., isolates from *B. pinnatum* relative to the main group), and *E. baconii* from *Agrostis* and *Calamagrostis* exhibit genetic identities of 0.5 or less (Fig. 4). Values of this magnitude are often typical for even distantly related species in other fungi (Keller, 1992) or in other organismal groups (Thorpe, 1983). The cause for such divergence and obvious genetic isolation of strains or whole populations may be their host specificity. Specificity of host strains was evident in *E. typhina* involving reciprocal inoculations of *D. glomerata* and *L. perenne* (Chung et al.,
1997), and was clearly suggested for isolates from A. odoratum and D. glomerata (Leuchtmann, unpublished data). Strong association with a host together with ecological or geographic mechanisms of isolation should provide a fertile ground for speciation to occur. Host specialization may have been a major factor for the speciation of E. sylvatica, which presumably emerged from ancestral E. typhina strains (Leuchtmann and Schardl, 1998).

Another mechanism which may lead to partial or complete isolation of different fungal populations is a difference in host flowering times, which is

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typically correlated with the emergence of stromata. Matings between different host strains can occur only when spermatia for fertilization and susceptible stromata are available at the same time. Also, horizontal transmission between hosts would be prevented if the route of infection is the exerted stigma, as postulated (Chung and Schardl, 1997). Early-flowering *A. odoratum* infected by *E. typhina* is an example where flowering time does not coincide with that of most other sympatric host species. This could reproducitively isolate the endophyte and may explain the considerable genetic divergence observed in strains from that host (Fig. 4).

Finally, *Botanophila* flies, the main vector of spermatia in heterothallic matings, could be a factor in sympatric isolation, if they were specialized to certain *Epichloë* associations. A case of such fly-mediated isolation may be the morphospecies *E. clarkii* on *H. lanatus*, which appears to maintain genetic and morphological distinction from *E. typhina*, although it is completely interfertile experimentally with that species. Recent observations in field plots based on ascospore progeny analysis after natural matings seem to support this notion (Leuchtmann and Bultman, 2001). However, other studies on the feeding behavior of *Botanophila* flies indicated that flies may not be specific, as will be discussed in more detail later.

### 3.5. Nonstromatal *Epichloë* Endophytes

There are several examples of asexual endophytes which obviously lost the capability to form stromata but still remain sexually compatible with their nearest presumed *Epichloë* ancestor. Although these endophytes may represent distinct incipient species, they are by convention classified under their respective sexual *Epichloë* species. Two of the better-known examples are discussed below.

#### 3.5.1. *E. Bromicola*

*E. bromicola* infects at least three species of *Bromus* (Leuchtmann and Schardl, 1998) and was recently identified also from *Hordelymus europaeus* (Leuchtmann and Moon, unpublished data). The different host strains are interfertile among each other (at least experimentally) and thus represent the same biological species, but they differ in their life cycle and in the type of symbiosis. Strains naturally infecting *B. erectus* are choke-forming during the sexual cycle and fail to transmit in host seed, whereas strains from *B. benekenii, B. ramosus*, and *H. europaeus* lack a sexual stage and are purely vertically transmitted by seed. Genetic analysis based on AFLP patterns of multiple isolates from the three *Bromus* hosts indicated that *E. bromicola* consists of several genetically divergent lineages which are restricted to a particular host and appear to be reproductively isolated (Brem, 2001). Moreover, the phylogenetic trees inferred from intron sequences of the tub2 and tef1 genes from these isolates suggested that all seed-transmitted strains...
were derived from ancestral, sexual lineages of *E. bromicola*. Therefore, strains infecting *B. benekenii* and *B. ramosus* appear to have diverged after the host shift and may represent one or several incipient species that have not yet completely developed genetic barriers to mating but are *de facto* reproductively isolated through loss of sexuality and adaptation to the new host. In reciprocal inoculation tests with plant seedlings, seed-transmitted isolates from *B. benekenii* and *B. ramosus* could easily be moved among the asymptomatic hosts, but not to *B. erectus*, indicating that they are host-specific, while the stroma-forming *E. bromicola* strains were broadly compatible with all three hosts (Brem, 2001). However, artificially infected *B. benekenii* and *B. ramosus* plants formed stromata only in the first year, and infection became lost after prolonged growth of the plants in the greenhouse. Under natural conditions and involving other genotypes, contagious infections may be more persistent.

Asexual strains of *E. bromicola* on *B. benekenii* and *B. ramosus* should be expected to show little genetic variation, due to their clonal growth. However, the genetic variation both of isozymes (Leuchtmann, 1994) and gene sequences (Brem, 2001) observed in asexual isolates from these hosts was considerable and more than one would expect from long-standing clonal lineages. It is possible that repeated host shifts occur and that simultaneous infections by seed-transmitted and sexual strains allows for low levels of gene flow resulting from somatic recombination in parasexual processes.

The emergence of the nonstromatal populations of *E. bromicola* apparently involved a change of the transmission mode from purely horizontal in sexual strains to purely vertical in asymptomatic strains. Interestingly, there is no intermediate stage with a balanced type of symbiosis known in *E. bromicola*, as in many other associations. Flowering culms occasionally formed on infected plants of *B. erectus* have escaped infection, and seeds are endophyte-free (personal observation). Thus, adapational changes on the new hosts included both loss of sexuality and the development of mechanism to grow into seeds. If seed transmission were an ancestral trait, reversal to that mode of transmission may have been simple, but if not, complex mutual adaptations in the tissue compatibility and relative growth speed of host and fungus were necessary. It is therefore possible that *E. bromicola* was either purely sexual or possessed both transmission modes initially after host shift and then lost the sexual cycle. Selective forces that promoted seed transmission may have been the increased mutualistic effects after restoring host fertility and the more efficient dissemination of the endophyte within seeds. Infected *B. benekenii* plants have superior competitive abilities compared to uninfected plants (Brem and Leuchtmann, 2002) and may be better protected from insect herbivores (Biber and Leuchtmann, unpublished data). The high level of infection of usually more than 80% found in natural populations of *B. benekenii* and *B. ramosus* (Leuchtmann, 1996) further reflects the increased success of seed-transmitted...
associations of *E. bromicola* which are no longer reliant on horizontal transmission. The loss of sexuality in seed-transmitted endophytes is also consistent with evolutionary models predicting that vertically transmitted parasites evolve toward less virulence (Lipsitch et al., 1995) (for further discussion of these issues, see Chapter 9, this volume).

### 3.5.2. *E. sylvatica*

In *E. sylvatica* infecting *Brachypodium sylvaticum*, nonstromatal forms obviously emerged from sexual strains which co-occur as two distinct subpopulations on the same host grass (Bucheli and Leuchtmann, 1996). One subpopulation consists of genotypes which are strictly stroma-forming and the other of genotypes which are asexual and seed-transmitted. As with host-associated *E. bromicola* strains, the two subpopulations of *E. sylvatica* do not appear to be panmictic, even though for the most part they still are sexually compatible in artificial mating tests. It is the difference in their life cycles which prevents outcrossing between the subpopulations in nature. Interestingly, artificial mating with purely asexual strains used as male parent tend to be less fertile compared to matings between stromata-forming genotypes, indicating that weak genetic barriers to mating may already exist (personal observation).

A remarkable and insufficiently explained attribute of *E. sylvatica* is the very high infection level of *B. sylvaticum* by nonstromatal strains, typically reaching 100%, and the wide distribution throughout the range of the host in temperate Eurasia. In most areas only asexual, seed-transmitted forms are present, often as single clones, whereas stroma-forming endophytes are rare and genetically more divers. Plants with mixed symptoms, previously considered to be a balanced host interaction, are simultaneously infected by different genotypes, one sexual and the other asexual (Meijer and Leuchtmann, 1999). However, sexual strains are capable of seed transmission as well when special circumstances allow for flowering of infected culms, which distinguish them from other stroma-forming *Epichloë* species such as *E. typhina*.

Associations of *B. sylvaticum* with asexual strains appear to have a strong selective advantage over uninfected plants, which resulted in the observed high levels of infection. However, it is not clear what the benefits of infection are for the host plants. Stimulatory effects on growth or improved competitive abilities which are well documented for other grasses have not been found with infected *B. sylvaticum* plants (Brem and Leuchtmann, 2002). However, increased resistance to herbivory could be a relevant factor. In laboratory experiments, the insect herbivore *Spodoptera frugiperda* performed significantly better on a diet of uninfected leaves of *B. sylvaticum* compared to the infected control, even though the chemicals causing this effect are not known (Brem and Leuchtmann, 2001). Moreover, in natural, entirely infected populations of the host grass, microherbivores caused considerably more damage to tillers bearing fungal
stromata than to asymptotically infected tillers. This suggests that asexual strains of *E. sylvatica* may be better able to protect their hosts against insect herbivory than sexual strains. Possible alternative explanations, which have not yet been explored in the *E. sylvaticum* system, for the high frequency of asexual strains may be the increased resistance to microbial pathogens (Clay, 1990; Kimmons et al., 1990; Gwinn and Gavin, 1992) or a better nutrient uptake induced by changed chemical environments near the root zone (Malinowski et al., 1998, 1999). Another intriguing idea is that seed-transmitted *E. sylvaticum* could alter the reproductive system of the host grass, such that seed production and hence endophyte transmission is increased. *Brachypodium sylvaticum* is the only selfing species of the genus, which allows for dependable and much higher production of fertile seed compared to the outcrossing species (Schippmann, 1991), and it is the only species known to be infected by a seed-transmitted endophyte. However, whether self-compatibility was induced by endophyte infection, or conversely was preexisting and then facilitated the success of this association, is not known and needs to be tested.

An interesting question is how sexual strains of *E. sylvatica* can coexist together with the asexual subpopulation on the same host and why they continue to persist. Like most successful pathogens, *E. sylvatica* has efficient means for contagious spread in the form of wind-dispersed ascospores. Up to one-third of endophyte-free *B. sylvaticum* plants placed within stroma-forming plants at natural sites became infected after 2 years, indicating that ascospores very frequently mediate new infections in tillers of adult plants, presumably through invasion of vegetative tissues or tiller buds (Brem and Leuchtmann, 1999). Horizontal transmissions in unmanipulated stands may actually be lower, because most plants are already infected and new strains have to compete with the resident endophyte in the host. In fact, recent findings suggest that infection by seed-transmitted endophytes cause host plants to become less susceptible to superinfection by choke-forming strains (Meijer and Leuchtmann, 2000). Multiple infections of single plants occur frequently in natural populations of *B. sylvaticum* (Meijer and Leuchtmann, 1999). However, genetically distinct strains, often with different symptom type, always occupy different parts of a plant, which may be the result of competitive displacement at the tiller level after superinfection. Based on evolutionary theory of host–parasite interactions (Hamilton, 1980; Bell, 1982), sexual reproduction of a parasite may be more important in less mutualistic interactions. Regular sexual recombination of the strictly stroma-forming strains of *E. sylvatica* enables them to overcome eventual resistance of host genotypes and to win the coevolutionary arms race between the asymptptomatically infected host plant and the stroma-forming parasite. The sexual subpopulation found today may have remained in a transitional stage toward mutualism with the capacity to form stromata and the potential for seed transmission.
4. THE FLY MUTUALISM
4.1. Life Cycle and Diversity of Botanophila Flies

*Epichloë* species have long been known to interact with small dipteran flies of the family Anthomyiidae (Giraud, 1872; Lucas, 1909). These fly species have in older literature been referred to *Phorbia* or *Pegohylemyia* (Ackland, 1972; Kohlmeyer and Kohlmeyer, 1974; Bultman, 1995), but should correctly be placed in genus *Botanophila* (Michelsen, 1985). The specialized *Botanophila* flies ingest and transfer spermatia of *Epichloë* while visiting stromata for oviposition (Bultman and White, 1988; Bultman et al., 1995). The transfer of spermatia involves a specific behavior during which the female flies stereotypically drag their abdomen across the stromal surface in a spiral pattern while defecating spermatia (Bultman et al., 1998). Spermatia pass the fly’s digestive tract intact and remain viable for cross-fertilization of the stroma. This active method of transfer obviously ensures that the whole stroma surface is efficiently fertilized. The result of fertilization can be seen on many naturally occurring stromata, where perithecia initially develop as distinct straight or spiraling lines which originate at the exact site of oviposition. The large sculptured eggs, typically one per visit, are deposited on the surface of the stroma. In some species, however, eggs are smooth and smaller and are inserted into stromal crevices or tissues of the infected grass. Flies subsequently visiting the stroma may also lay eggs, so that several eggs (up to seven) accumulate on the same stroma over 1–2 weeks (Bultman et al., 2000). Upon hatching, larvae feed and develop on the fertilized stromata by consuming fungal tissues including perithecia and ascospores. In the course of development, larvae of most species construct a tubelike brood chamber, with the egg shell on top of the tube, from which they depart periodically for feeding. Other species burrow away from the egg shell slightly below the surface of the stroma soon after hatching and then make a long silken tube inside (Ackland, 1972). Eventually, both types of larvae borrow inside the choked grass stem and remain there until they drop to pupate in the soil beneath the grass clump. From there they emerge as adults the following spring.

It appears that *Botanophila* flies and *Epichloë* fungi live in a delicately balanced mutualistic symbiosis analogous to parasitic insects that pollinate flowering plants (Bultman, 1995). The fungi benefit from the adult flies as reliable vectors of spermatia for cross-fertilization, while fly larvae depend on the fertilized stromata as food source. Thus, the motive of the fly to actively fertilize the fungal stroma is provisioning of its offspring. Only fertilized stromata produce abundant perithecial tissue on which fly larvae feed. Since larvae can consume a substantial part of the perithecia produced, the mutualism may also impose a cost to the fungus by reducing the potential output of ascospores, the propagules for contagious spread (Welch and Bultman, 1993). Overexploitation
of the fungus by the fly could outweigh the benefit of fertilization. However, the interaction appears to be stable. An explanation for why flies do not overexploit Epichloë stromata was recently provided by Bultman et al. (2000), who observed that greater fly visitation with multiple eggs laid on a single stroma resulted in increased larval mortality. The causes for mortality are unknown, but could be due to diseases or parasitoids which are commonly found to emerge from Botanophila pupae (Kohlmeyer and Kohlmeyer, 1974, B. Merz, personal communication).

At least five different Botanophila species are reported to be associated with Epichloë hosts in Europe, and different Botanophila species appear to coexist at the same locality or even on the same host fungus (Collin, 1967; Hennig, 1976; Michelsen, 1985). Confirming such observations, a recent survey of Botanophila larvae feeding on Epichloë stromata in Switzerland found four distinct larval types based on DNA sequence data (Table 2). Although it was not possible to link larval types with known Botanophila species, except for B. lobata, the amount of sequence divergence suggested that presumably all represented distinct fly species. Identification of Botanophila species can be done with certainty only on characters found in adult male flies. These do not visit the fungal stromata and are difficult to find. The most common fly taxon was found to be associated with six different Epichloë species infecting 12 grass hosts, while the others appeared to have a more restricted host range (Table 2). Moreover, all four fly taxa sometimes occurred together in a host community at the same site, with up to three fly taxa associated with the same host grass. Presumed fly taxa

<table>
<thead>
<tr>
<th>Taxon 1</th>
<th>Taxon 2</th>
<th>Taxon 3</th>
<th>Taxon 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Epichloë spp.</td>
<td>6</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>No. of host grasses</td>
<td>12</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Time of appearance</td>
<td>Early/late</td>
<td>Early/late</td>
<td>Late</td>
</tr>
<tr>
<td>Locations</td>
<td>Zumikon</td>
<td>Zumikon</td>
<td>Zumikon</td>
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<td></td>
<td>Abegg</td>
<td>Abegg</td>
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<tr>
<td></td>
<td>Vaud</td>
<td>Vaud</td>
<td>Vaud</td>
</tr>
</tbody>
</table>

*Species included E. baconii, E. bromicola, E. clarkii, E. festucae, E. sylvatica, E. typhina.*

*Hosts included Agrostis tenuis, Anthoxanthum odoratum, Brachypodium pinnatum, B. sylvaticum, Bromus erectus, Dactylis glomerata, Festuca rubra, Holcus lanatus, H. mollis, Phleum pratense, Poa nemoralis, P. trivialis.*
differed in their time of appearance. Two taxa stayed active with egg laying during at least 6 weeks starting in late April, while others appeared later in May or early June.

Preliminary sequence data from *Botanophila* larvae collected in the southeastern United States suggested that additional fly taxa may occur on the American continent (Leuchtmann, unpublished results).

### 4.2. Are *Botanophila* Flies Specialized?

With multiple *Botanophila* species and multiple hosts frequently present in an area, an obvious question is whether flies are specialized to particular hosts. From an evolutionary point of view, specialization to a single *Epichloë* species or mating populations should be selectively advantageous, because fly larvae depend on successfully fertilized stromata as food source, a process which may be facilitated if only compatible spermatia are transferred. Similar patterns of specialization are known from pollinating parasites of flowering plants, notably fig wasps associated with their fig hosts (Bronstein, 1992). Observations made in experimental field plots containing different infected host grasses of *E. tephina* and *E. clarkii* side by side suggested that matings, presumably mediated by *Botanophila* flies, occurred only among stromata of the same host species (Leuchtmann and Bultman, 2001). These observations were based on parental analysis of ascospore progeny collected from both mating types of *E. clarkii*. However, further analyses performed on ascospore samples made in the following year indicated that natural cross-fertilizations may also occur among stromata of different hosts, notably between *E. clarkii* infecting *Holcus lanatus* and *E. tephina* infecting *Anthoxanthum odoratum* (Leuchtmann, unpublished data). These interspecific matings must have been initiated by flies which visited both hosts before defecating spermatia onto the cross-fertilized stromata. Indeed, genetic analysis of spermatia present in the faeces of field-caught flies revealed that most individuals carried a mixture of spermatia of up to four different genotypes in their gut (Leuchtmann and Bultman, 2001). These genotypes represented *Epichloë* species of different hosts as well as of different mating populations. *Botanophila* flies appear to have foraged on several hosts that were available and thus may not be specific to individual hosts. However, flies may be adapted to native hosts in different continents. In 4 years of observation at a field plot in Kentucky, USA, the native *E. elymi* has been visited and mated by flies while *E. festucae* and *E. tephina*, infecting introduced grasses indigenous to Europe, were ignored (Scharld and Leuchtmann, 1999).

The apparently unspecific behavior of the flies could have consequences for the reproductive success of fertilized stromata. Spermatia from all *Epichloë* species (mating populations) can induce the formation of perithecia if they are of opposite mating type at least under experimental conditions, but perithecia which
are the result of interspecific matings remain barren and will not produce ascospores (Chung and Schardl, 1997). Such potentially abortive matings could suppress or at least reduce fertility of stromata in nature. However, barren perithecia have never been observed in natural matings mediated by flies. Thus, mechanisms may exist which prevent unwanted matings of the stromata or favor fertilizations with spermatia from the own mating population. It is possible that spermatia deposited by flies in a mixture differ in their competitiveness to fertilize its own or a different species. Supporting this notion, experimental mating tests using spermatia from different *Epichloë* indicated that interspecific mating interactions mostly did not preclude subsequent intraspecific matings (Chung and Schardl, 1997).

### 4.3. Mechanisms of Host Recognition

Most *Epichloë* stromata emanate a distinct smell, particularly when they have emerged freshly or are wet. This smell may selectively attract *Botanophila* flies to feed and to oviposit on the stromata. Behavioral studies with manipulated and unmanipulated stromata suggested that the fungal odor may be the primary stimulus for depositing eggs (Kohlmeyer and Kohlmeyer, 1960). A possible role of volatile components of the odor for the attraction of flies over long distances has also been considered, but could not be verified experimentally by these authors. It was therefore concluded that flies may not need to fly long distances to find fungal stromata, because they typically emerge from pupae beneath the infected host. However, infected grasses that have been transplanted to a new site where no *Epichloë* was present within at least 1 km were readily visited by flies the following spring (Kohlmeyer and Kohlmeyer, 1960). This would argue for long-distance attraction of flies by the fungus. Similar observations were also made recently in experimental plots with multiple host species of *Epichloë* in which few of the stromata remained undetected by flies 1 year after transplanting (Leuchtmann, personal observation). Moreover, genetic analysis of ascospore progeny indicated that fertilization often occurred with unknown spermatia from outside the experimental plot, further supporting long-range activity of flies (Leuchtmann, unpublished data).

Analysis of volatile fragrances that were produced by *Epichloë* stromata and trapped by using Poropaq Q showed surprising diversity (Roy and Leuchtmann, unpublished data). Each profile of volatiles found in samples from freshly collected stromata of five different *Epichloë* species appeared to be distinct. In *E. typhina*, profiles were even distinct for each of four hosts of that species. Many of the volatiles belonged to compound classes, including sesquiterpene alcohols and fatty acid derivatives, which are frequently found in insect-attracting fragrances of flowering plants (Dobson, 1994). This makes it likely that the odor of *Epichloë* stromata serves to attract insects. I hypothesize
that volatiles are important cues for attracting *Botanophila* flies over long distances and that some may be selectively attractive to different fly species. However, this hypothesis needs to be further tested experimentally. Over short distances, the conspicuous white color of young *Epichloë* stromata may additionally guide insects to the stromata.

5. CONCLUSIONS

Diversity of *Epichloë* endophytes is the result of very intimate and complex interactions among fungus, grass host, and fertilizing insects together with the environment. A key factor in the evolution of the genus appears to be the variation in the modes of transmission, which are believed to be linked with the degree of mutualism versus antagonism of an association. Although it remains controversial which type of symbiosis may represent the ancestral state, molecular evidence indicates that in the course of radiation of the genus evolution has moved in both directions several times. Host-related genetic diversity in some biological species suggests that they consist of several reproductively isolated subpopulations. Mechanisms of isolation may include host specificity and differences in host flowering time. Among nonstromatal *Epichloë* endophytes which are not of hybrid origin and closely related to sexual species, incipient speciation may be common and occurs (through host shifts, host specialization, and change of the reproduction mode) long before intersterility among species has evolved.

*Epichloë* species maintain a symbiotic interaction with *Botanophila* flies, which play a crucial role in the sexual cycle as vector of gametes (conidia). Several fly species are involved which may co-occur at the same site. Although there is some evidence of preference, most flies appear to visit and feed on multiple hosts and thus do not control mating specificity of *Epichloë* species. It is assumed that volatile fragrances of fungal stromata specifically attract *Botanophila* flies over long distances, while the conspicuous white color of young stromata guides insects to find hosts on short range.

ACKNOWLEDGMENTS

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Diversity and Speciation in *Claviceps*

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1. **INTRODUCTION**

The genus *Claviceps* includes fungi specialized in the parasitism of the ovaries of grasses and a few species of rushes and sedges. Unfertilized ovaries are especially susceptible to infection. *Claviceps* species produce stipitate, spherical stromata. Perithecia are at least partially embedded within the stromata and are distributed over the surface, resulting in a punctate appearance. Thin, filiform-shaped ascospores are forcibly ejected and become airborne. Ascospores that land on stigmas of a susceptible host germinate and produce infection hyphae that grow down the element to infect the base of the ovary. Within several days of infection, a sphaecelium producing large numbers of conidia develops. A sugary syrup derived from plant sap, along with conidia, ooze from infected florets in what is commonly referred to as the honeydew stage. Conidia are also infective and are responsible for secondary spread. The honeydew stage is replaced by a sclerotium, generally 1–4 times larger than the host seed. The sclerotium is the structure most easily recognized and associated with *Claviceps* infection, especially for the common and widespread species, *C. purpurea* var. *purpurea*. Sclerotia serve as the survival structure of *Claviceps*, allowing the fungus to survive periods when susceptible hosts are not receptive to infection (flowering).
Germination of sclerotia and subsequent development of stromata and release of ascospores, typically coincides with flowering in the host plant. Morphological characteristics that can vary among species of *Claviceps* include: color and morphology of the sclerotium; color of the stipe and capitulum; presence or tufts of mycelium at the base of the stipe; presence of an annulus below the capitulum on the stipe; size and shape of the perithecia, asci, and ascospores; the density and extent of projection of the perithecia on the outer surface of the capitulum; size and shape of macroconidia, microconidia or secondary conidia; and host range.

As pathogens primarily of grasses, the geographical distribution of *Claviceps* species parallels the wide distribution of grasses, extending from the tropics to the sub-Arctic, and including environments ranging from semiarid to marine. Most *Claviceps* species are restricted to a single host genus or closely related genera. The ecological niche for *Claviceps* is the flower, the ovary in particular, and *Claviceps* species have co-evolved with grasses in such a way that the reproductive/host-infecting phase of the fungus coincides with the reproductive/flowering period of the host plant. *Claviceps* species have also adapted to specific environments, which regulate survival, stimulation of germination, spore release, secondary spore production and dissemination, and subsequent production of sclerotia. Thus, evolution and diversity of *Claviceps* have occurred through the combination of specific host and environmental constraints.

The genus *Claviceps* falls within the family Clavicipitaceae (Hypocreales). Diehl (1950) recognized three subfamilies, Oomycetoideae, Clavicipitoideae, and the Cordycipitoideae, and emphasized further division of the Clavicipitoidae into three tribes, Clavicipiteae, Balanseiae, and Ustilaginoideae, based on fundamental differences in conidiation. In the Clavicipiteae, represented by *Claviceps*, there is a distinctive sphacelial stage. In the Ustilaginoideae there are smutlike spores borne on closely packed parallel hyphae. In the Balanseiae the conidiation is typically ephelidial or may be lacking. However, the lines between these groups are not entirely distinct, especially among tropical species, where some species appear to be intermediary between genera. Even within the genus *Claviceps*, demarcation between species is not entirely clear, due to variability in host range within and among species and in an incomplete understanding of the morphological variability within species. Additional studies, particularly molecular phylogenetics, will be required to better characterize and determine the taxonomic placement of genera and species within the Clavicipitaceae.

In this chapter an attempt is made to address diversity in the genus *Claviceps* in terms of morphology, host range, and biology. A summary of morphological and biological characteristics for each of the known species of *Claviceps* is provided. Technical descriptions for each species are condensed to include details most relevant for comparative purposes. A summary of morphological features helpful in separation of species is provided in Table 1. For some species, changes in synonymies are proposed.

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<table>
<thead>
<tr>
<th>Species</th>
<th>Sclerotia</th>
<th>Stipe</th>
<th>Capitulum</th>
<th>Conidia</th>
<th>Host genera</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. africana</td>
<td>Spherical, reddish brown</td>
<td>Translucent, then purple, glaborous</td>
<td>Dark purple</td>
<td>Macro: 9–17 × 5–8 μm, oblong to oval; Micro: 2–3 μm, spherical; Secondary: 8–14 × 4 – 6.5 μm, pear-shaped</td>
<td>Sorghum, Zea, Pennisetum</td>
</tr>
<tr>
<td>C. amamiensis</td>
<td>Cylindrical with blunt or tapered apex, straight or curved, blackish-purple or black</td>
<td>Light brownish purple; no tuft at base</td>
<td>Dark purple</td>
<td>Macro: 9.8–14.6 × 2.0–4.1 μm, fusoid to allantoid; Micro: 2.4–5.6 × 2.0–3.5 μm, elliptical–subglobose</td>
<td>Digitaria</td>
</tr>
<tr>
<td>C. annulata</td>
<td>Cylindrical elongated, tapered at apex, chestnut to blackish brown</td>
<td>Amber, annulus below capitulum</td>
<td>Chestnut</td>
<td>8.5–12.5 × 3.5–6 μm, oblong</td>
<td>Eulalia</td>
</tr>
<tr>
<td>C. balansoides</td>
<td>Mycelial-scleotial, blue-black</td>
<td>Bright yellow</td>
<td>Bright yellow</td>
<td>9–12 μm</td>
<td>Panicum</td>
</tr>
<tr>
<td>C. bothriochloae</td>
<td>Cylindrical to clavate, taper at apex, dark brown to dark purple</td>
<td>Sulfur</td>
<td>Sulfur</td>
<td>2.6–5.9 × 1.8–3.2 μm, elliptical or oval</td>
<td>Bothriochloa</td>
</tr>
<tr>
<td>C. cinerea</td>
<td>Clavate, tapered towards apex, gray</td>
<td>White</td>
<td>Light gray</td>
<td>Broadly fusiform, slightly curved, spherical</td>
<td>Hilaria</td>
</tr>
<tr>
<td>Species</td>
<td>Sclerotia</td>
<td>Stipe</td>
<td>Capitulum</td>
<td>Conidia</td>
<td>Host genera</td>
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<tr>
<td><em>C. citrina</em></td>
<td>Oblong to clavate, straight or slightly curved, brown to gray</td>
<td>Pale yellow</td>
<td>Lemon yellow</td>
<td>3.65–7.2 × 2.5–2.7 µm, elliptical</td>
<td><em>Distichlis</em></td>
</tr>
<tr>
<td><em>C. cynodontis</em></td>
<td>Fusiform, straight or curved, deep brown</td>
<td>Lucid</td>
<td>10–20 × 4–6 µm, reniform</td>
<td></td>
<td><em>Cynodon</em></td>
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<tr>
<td><em>C. cyperi</em></td>
<td>Cylindrical straight, dark brown to near black</td>
<td>Pale straw, swollen at base</td>
<td>Pale straw, collar below</td>
<td>5.5–13 × 2–4 µm, oblong–elliptical</td>
<td><em>Cyperus</em></td>
</tr>
<tr>
<td><em>C. digitariae</em></td>
<td>Oval to oblong, dark brown to near black</td>
<td>Yellow to yellowish white, white tuft at base</td>
<td>Yellow with purple ostioles, collar at base</td>
<td>10.5–17.5 × 3–5.5 µm, elliptical</td>
<td><em>Digitaria</em></td>
</tr>
<tr>
<td><em>C. fusiformis</em></td>
<td>Pyriform to obpyriform, light brown to blackish brown</td>
<td>Pale purple or cream</td>
<td>Grayish purple, slightly papillate</td>
<td>Macro: 12–26.4 × 2.4–6 µm, broadly fusiform; Micro: 2.4–10.8 × 1.2–4.8 µm</td>
<td><em>Sorghum, Panicum, Setaria</em></td>
</tr>
<tr>
<td><em>C. gigantea</em></td>
<td>Comma-shaped, white to grayish brown</td>
<td>Pink to red dish brown</td>
<td>Pink ostioles, slightly papillate</td>
<td>Macro: 8.3–27 × 4.2–5.8 µm, elliptical to fusiform; Micro: 4.2–6.7 × 2.5–3.3 µm</td>
<td><em>Zea</em></td>
</tr>
<tr>
<td><em>C. glabra</em></td>
<td>Subglobose, black</td>
<td>Pale straw-yellow to cream</td>
<td>Purple red to chestnut</td>
<td>12.5–20.5 × 4.0–7.0 µm</td>
<td><em>Digitaria</em></td>
</tr>
<tr>
<td>Species</td>
<td>Description</td>
<td>Color Description</td>
<td>Size</td>
<td>Genus</td>
<td></td>
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<tr>
<td>C. grohii</td>
<td>Semicylindrical flattened on one side, straight or curved, blackish violet to blackish purple</td>
<td>Blackish violet to blackish brown, violet tuft at base</td>
<td>10–16 × 3–5 μm, arcuate</td>
<td>Carex</td>
<td></td>
</tr>
<tr>
<td>C. hirtella</td>
<td>Subglobose, yellowish to deep brown</td>
<td>Grayish pink, glabrous, white tuft at base</td>
<td>11.0–16.5 × 4.5–6.5 μm, elliptical, curved</td>
<td>Brachiaria, Eriochloa</td>
<td></td>
</tr>
<tr>
<td>C. imperatae</td>
<td>Cylindrical, ovoid, or subglobose, straight or curved</td>
<td>Brownish purple to reddish gray, purple tuft at base</td>
<td>6.7–27.5 × 3.7–9.2 μm</td>
<td>Imperata</td>
<td></td>
</tr>
<tr>
<td>C. inconspicua</td>
<td>Cylindrical or fusoid, dark brown to black</td>
<td>Anthrocene purple</td>
<td>15–20 × 5–10 μm, oblong, straight or curved</td>
<td>Hyparrhinia</td>
<td></td>
</tr>
<tr>
<td>C. lutea</td>
<td>Cylindrical bulge above lemma and palea, yellow</td>
<td>Bright yellow</td>
<td>9 × 12 μm</td>
<td>Paspalum</td>
<td></td>
</tr>
<tr>
<td>C. maximensis</td>
<td>Straight or slightly curved, tapered towards apex, brown to gray brown</td>
<td>Pale green, then yellow</td>
<td>10–30 × 3.5–11 μm, elliptical</td>
<td>Panicum</td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Sclerotia</td>
<td>Stipe</td>
<td>Capitulum</td>
<td>Conidia</td>
<td>Host genera</td>
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</tr>
<tr>
<td><em>C. microspora</em></td>
<td>Cylindrical, tapered, blackish purple or black</td>
<td>Dark purple or light black, no tuft at base</td>
<td>Dark purple or light black with dark purple ostioles</td>
<td>2.2–3.8 × 2.0–2.9 μm, ellipsoid to subglobose</td>
<td><em>Arundinella</em></td>
</tr>
<tr>
<td><em>C. nigricans</em></td>
<td>Cylindrical with rounded or tapered apex, purplish black</td>
<td>Bluish violet</td>
<td>Cinnamon buff then near black</td>
<td>8–12 × 3–4 μm, oblong</td>
<td><em>Eleocharis</em></td>
</tr>
<tr>
<td><em>C. orthocladae</em></td>
<td>Subglobose, mycelial-clerotial, yellow</td>
<td>Yellow</td>
<td>Yellow, ostioles prominent</td>
<td>2.5–5.9 × 1.6–2.5 μm, ellipsoid or ovid</td>
<td><em>Orthoclada</em></td>
</tr>
<tr>
<td><em>C. panicoide-arum</em></td>
<td>Oblong, ovoid, or obclavate, dark purple or black</td>
<td>Blackish purple, tuft at base</td>
<td>Blackish purple, dark purple ostioles</td>
<td>8–16 × 4–7 μm, oblong</td>
<td><em>Ischne</em>, <em>Miscanthus</em></td>
</tr>
<tr>
<td><em>C. paspali</em></td>
<td>Globose, yellow to gray</td>
<td>White</td>
<td>White, then yellow, finally brown</td>
<td>5.5–15.0 × 2–4 μm, oblong, straight or slightly curved, tapered</td>
<td><em>Paspalum</em></td>
</tr>
<tr>
<td><em>C. phalaridis</em></td>
<td>Spherical to oblong, fawn</td>
<td>White, then purple drab to dark purple drab</td>
<td>Dark purple to black</td>
<td></td>
<td><em>Phalaris</em></td>
</tr>
<tr>
<td>Species</td>
<td>Description</td>
<td>Color</td>
<td>Length</td>
<td>Width</td>
<td>Genus</td>
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</tr>
<tr>
<td>C. platytricha</td>
<td>Cylindrical straight or curved, dark chestnut</td>
<td>Umber, glabrous</td>
<td>7.5–11 µm</td>
<td>3.5–5.5 mm</td>
<td>Ischaemum</td>
</tr>
<tr>
<td>C. purpurea var purpurea</td>
<td>Cylindrical with rounded or tapered apex</td>
<td>Cream to purple, tuft at base</td>
<td>4–6 × 2–3 µm, elliptical, larger in some strains</td>
<td>8.06 ± 1.8×4.12 ± 1.3 µm</td>
<td>Numerous, mostly in Pooideae</td>
</tr>
<tr>
<td>C. purpurea var spartinae</td>
<td>Cylindrical tapered towards apex, purple brown to dark brown</td>
<td>Purple brown, White to slightly tan, with reddish ostioles</td>
<td>10.0–15.5×5.0–7.5 µm, triangular, some elliptical</td>
<td></td>
<td>Spartina</td>
</tr>
<tr>
<td>C. pussila</td>
<td>Cylindrical, brown to black</td>
<td>Pale straw, white tuft at vase</td>
<td>10–20 × 3.5–5 µm, oblong, almost cuneate</td>
<td></td>
<td>Numerous, mostly in Andropogoneae</td>
</tr>
<tr>
<td>C. queenslandica</td>
<td>Pale yellow</td>
<td>Yellow</td>
<td>7–8 × 3 µm, oval</td>
<td></td>
<td>Paspalum</td>
</tr>
<tr>
<td>C. ranunculoides</td>
<td>Cylindrical, tapered at apex, blue-purple</td>
<td>Bright yellow, tuft at base</td>
<td></td>
<td></td>
<td>Setaria</td>
</tr>
<tr>
<td>C. rhynchelytri</td>
<td>Ellipsoidal, dark brown</td>
<td>Straw</td>
<td>5.5–10.5 × 2.5–4.5 µm, reniform</td>
<td></td>
<td>Rhynchelytrum</td>
</tr>
<tr>
<td>C. sorghi</td>
<td>Cylindrical, straight or curved, cream to buff, then light brown</td>
<td>Bronze or deep terracotta, Buff with white collar below</td>
<td>8–19 × 4–6 µm, oblong to oval, Micro: 2–5 µm, spherical</td>
<td></td>
<td>Sorghum, Sehima, Dicanthium, Ischaemus</td>
</tr>
<tr>
<td>Species</td>
<td>Sclerotia</td>
<td>Stipe</td>
<td>Capitulum</td>
<td>Conidia</td>
<td>Host genera</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------------------------</td>
<td>----------------</td>
<td>---------------</td>
<td>--------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td><em>C. sorghicola</em></td>
<td>Cylindrical to conical, straight or curved, purple black to black</td>
<td>Brown to bronze</td>
<td>Dark brown</td>
<td>5–11.3 × 2.5–3.8 μm, ellipsoidal to oval</td>
<td><em>Sorghum</em></td>
</tr>
<tr>
<td><em>C. sulcata</em></td>
<td>Elongated, straight or curved, slightly flattened, bifurcate, gray brown to near black</td>
<td>Bright orange then pale orange, tuft at base</td>
<td>Straw or bright orange</td>
<td>7.5–19 × 3–5 μm, allantoid or elliptic</td>
<td><em>Brachiaria</em></td>
</tr>
<tr>
<td><em>C. tripsaci</em></td>
<td>Conical, white, then brown</td>
<td>Whitish purple</td>
<td>White to gray</td>
<td>17.4–37.7 × 2.0–8.7 μm, fusoid to lunulate</td>
<td><em>Tripsacum</em></td>
</tr>
<tr>
<td><em>C. uleana</em></td>
<td>Cylindrical, dark brown</td>
<td>Gray to flesh Yellow or light olivaceous, no tuft at base</td>
<td>Gray to flesh Yellowish green to yellowish brown</td>
<td>Macro: 8.0–17.3 × 2.6–4.1 μm, bacilliform Micro: 3.5–6.7 × 2.3–3.3 μm, ovate</td>
<td><em>Panicum</em> <em>Brachypodium</em>, <em>Oplismenus</em></td>
</tr>
<tr>
<td><em>C. viridis</em></td>
<td>Cylindrical, blackish purple with greenish tint</td>
<td>Light olive-eous, no tuft at base</td>
<td>Yellowish green to yellowish brown</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. yanagawensis</em></td>
<td>Depressed cylindrical, curved</td>
<td>Dark brown</td>
<td>Pale red then dark purple to brown black</td>
<td>11.0–5.4 μm, fusoid</td>
<td><em>Zoysia</em></td>
</tr>
<tr>
<td><em>C. zizaniae</em></td>
<td>Light drab to brown</td>
<td>Tawny to light</td>
<td>Tawny to russet</td>
<td>6.5–30 × 3–6 μm, ellipsoidal, reniform</td>
<td><em>Zizania</em></td>
</tr>
</tbody>
</table>
2. SPECIES OF CLAVICEPS

2.1. *C. africana* Frederickson, Mantle & De Milliano

In Africa, *Claviceps* was known to occur on *Sorghum* in Kenya as early as 1924. Originally thought to be *Sphacelia sorghi*, the *Claviceps* was proven to be a species distinct from *S. sorghi* and is now recognized as *C. africana* (Frederickson et al., 1991). During the 1960s, coincident with introduction of hybrid sorghum production, *Claviceps* became a serious disease problem, especially on male sterile lines. The disease was strongly associated with cytoplasmic male sterility but only slightly associated with female sterility (Futrell and Webster, 1965). However, pollination of male sterile lines at the time they become receptive significantly reduces the level of ergot infection (Futrell and Webster, 1965). Thus, factors that interfere with pollination, e.g., low temperatures (<16°C) 3–4 weeks prior to flowering, increases disease susceptibility (McLaren and Wehner, 1992; McLaren and Flett, 1998; McLaren, 1997). High relative humidity (RH) is required for infection (Futrell and Webster, 1966).

Three days after inoculation with a conidial suspension, a superficial mycelial growth appears at the proximal end of the ovary (Bandyopadhay et al., 1990). Within 5 days of inoculation a fungal sphaecelium develops (Bandyopadhay et al., 1990). Honeydew appears within 7 days of inoculation (Frederickson et al., 1991). In *C. africana*, macroconidia on the surface of honeydew can germinate to produce conidiophores and secondary conidia, slightly smaller than the macroconidia (Frederickson et al., 1991). Production of secondary conidia occurs shortly after honeydew formation and is favored at 14–28°C with 12–16 h/day with RH above 90% (Bandyopadhay et al., 1990)). In spore-trapping studies, conidia were airborne from morning through midnight, with peak collection around 6:00 p.m. (Frederickson et al., 1989). In addition to secondary spread by airborne conidia, flies, bees, and other insects can facilitate disease spread (Futrell and Webster, 1966).

Whereas production of conidia is favored by high humidity (at least in the early stages of honeydew formation), sclerotial production is favored at 28–35°C and RH below 90% for 22 h/day (Bandyopadhay et al., 1990). Under field conditions, florets that became infected at the end of the wet season and develop in the dry season produce sclerotia free of disease (Futrell and Webster, 1966). Under prolonged periods of high humidity or during wet seasons, the honeydew is colonized by species of *Fusarium*, *Cerebella*, or *Cladosporium*, which interfere with subsequent production of sclerotia (Bandyopadhay et al., 1990; Futrell and Webster, 1966).

Sclerotia of *C. africana* are oval to spherical, with a reddish brown cortex patchily covered with a whitish sphaecelium. In moist sand at 20–25°C, sclerotia germinate after 4 weeks and mature after about 6 weeks, although they are susceptible to decay under moist conditions (Frederickson et al., 1991). Stipes are
glabrous, initially translucent, becoming purple. Capitula are dark purple, sub-globose, 0.5–1.3 mm. Perithecia are 86–135 × 123–226 μm. Asci are 140–3.2–4.2 μm with ascospores up to 45 × 0.8–1.2 μm. Paraphyses are absent at maturity. Macroconidia are oblong to oval, slightly constricted at the center, 9–17 × 5–8 μm (Fig. 1a). Microconidia are spherical, 2–3 μm in diameter (Fig. 1b). Secondary conidia are pear-shaped, 8–14 × 4–6.5 μm (Fig. 1c) (Frederickson et al., 1991).

The sclerotia contain ergoline alkaloids, mainly dihydroergosine, but also festucalvina, dihydroelymoclavine, and chanoclavine (total alkaloid 0.2–0.5%) (Frederickson et al., 1991). In cross-inoculation studies, Futrell and Webster (1966) demonstrated that conidia of C. africana from sorghum could infect Zea mays L. and Pennisetum glaucum (L.) R. Br. (= P. typhoides (Burm.) Stapf and C. E. Hubb.), and suggested that P. glaucum could represent a source of inoculum for Sorghum.

The ability of C. africana to produce a conidial phase that is readily transmitted through the air is an important development in the evolution of Claviceps. This provides very rapid disease spread within susceptible cultivars of sorghum (Frederickson et al., 1993). The true potential for disease spread was demonstrated when C. africana spread through sorghum production regions globally over the past two decades. The disease appeared in many African countries and in Thailand during the 1980s, and in Australia, South America, and into North America during the 1990s (Bandyopadhyay et al., 1998). Pazoutova et al. (2000b) identified two clades of C. africana, based on ITS and RAPD analysis. Both originated in Africa, but one group migrated to America and the other to Australia and India. Although other species of Claviceps may produce secondary conidia, C. africana is the only species in which the epidemiological role of the secondary conidia has been demonstrated.

2.2. C. amamiensis Tanda

C. amamiensis is known only from Digitaria microbachne Henr. in Japan (Tanda, 1992a). The sclerotia are straight or somewhat curved, 1.2–5.6 mm × 0.4–1.0 mm, and blackish purple or black. Stipes are light brownish purple with no tuft of mycelium at the base. The capitula are dark purple with perithecia immersed. Perithecia are 172–247 × 84–126 μm and ascospores are 91–133 μm. Macroconidia are fusoid or allantoid, 9.8–14.6 × 2.0–4.1 μm, and microconidia are ellipsoid or subglobose, 2.4–5.6 × 2.0–3.5 μm. The description for C. amamiensis is very similar to that of C. glabra, which occurs on Digitaria longiflora Pers. in Australia. Additional studies are needed to determine if the two collections represent distinct species. In addition, as suggested by Tanda (1992a), it is possible that a Claviceps from Digitaria ciliaris (Retz.) Koel. (= D. adscendens (Kunth) Henr.) in Formosa, which Swada (1944) designated as C. syntherismae (based only on the sclerotial state), is conspecific with C. amamiensis.
Figure 1  Variation in morphology of conidia among species of Claviceps. C africana macroconidia (a), microconidia (b), secondary conidia (c); C cinereum macroconidia (d), microconidia (e); C cyperi (g); C digitariae (h); C fusiformis macroconidia (i), microconidia (j); C gigantea macroconidia (k), microconidia (l); C grohii (m); C maximensis (n); C microspora (o); C nigricans (p); C panicoidearum (q); C paspali (r); C purpurea var. purpurea (s); C pusilla (t); C rhynchelytri (u); C tripsaci (v).
2.3. **C. annulata** Langdon

*Claviceps annulata* was described by Langdon (Langdon, 1942, 1952a), based on collections from *Eulalia fulva* (R. Br.) Kuntze near Dalby, Queensland, Australia. Sclerotia are cylindrical, chestnut or brown in color. A gray stroma is produced that differentiates into an amber-colored stipe and chestnut-colored capitulum. Langdon (1942) believed a ring of loose hyphae that develops at the base of the capitulum was characteristic of the species. Perithecia are 150–165 × 130–150 μm. Conidia are 8.5–12.5 × 3.5–6 μm, with sides parallel or converging and ends rounded (Langdon, 1942). However, in a later study, Langdon (1950a) mentions that *C. annulata* (as well as *C. inconspicua*) is similar to *C. pussila* and that it is possible that *C. annulata* could be an Australian variant of *C. pussila*. Given the wide geographic and host distribution of *C. pussila*, this is a possibility, although additional study is needed to determine their relatedness. *C. annulata* is known only from Australia.

2.4. **C. balansoides** Möller

In 1901, Möller described a fungus infecting a *Panicum* in Brazil that appears to be an intermediate between *Balansia* and *Claviceps*. During infection, the fungus develops within and among the flower to the extent that the fertile flower or both fertile and unfertile flowers are engulfed in a dense mycelial growth, as would be expected from development of *Balansia*. Conidia, 9–12 μm long, are produced in a whitish coating on the surface. Möller (1901) states that the flowering parts are not consumed but rather surrounded by the mycelium. Unfortunately, it is not clear to what extent the ovary is parasitized, and details of conidiation are not provided. A blue-black rind develops beneath the conidial layer. When Möller (1901) placed sclerotia on moist sand in May or June, they germinated by September and germination continued through December. Stipes and capitula are bright yellow in color. The stipes grow to lengths of 8 cm and capitula enlarge to 1.5 mm in diameter. Perithecia are 300 μm long. Asci are 150–180 × 3 μm, with a small cap. Upon germination, ascospores swell, become septate, and germinate to produce tapering conidiophores at the ends of which are produced a 12 × 15–μm conidia. The conidia germinate to produce conidia bearing mycelia. Although sharing features of both *Claviceps* and *Balansia*, Möller (1901) believed it to be more closely aligned with *Claviceps* than *Balansia*.

Petch (1933) believed that material assigned as *C. flavella* was conspecific with *C. balansoides*. However, the material examined by Petch has met with considerable controversy (Petch, 1933; Lloyd, 1920). More important, Petch’s description of *C. flavella* is not consistent with Möller’s description of *C. balansoides*. Petch (1933) describes the sclerotium as dark reddish brown with a red-brown cortex and bearing dark brown capitula on red-brown stipes. Even under conditions of storage, such drastic changes from yellow to dark brown...
would not be expected. It is more likely that the material examined by Petch (1933) is distinct from than that described by Möller (1901) (see additional discussion under *C. flavella*). Skalicky and Stary (1962) also recognized *C. balansoides* as distinct species but excluded it from their list as *Claviceps*, suggesting it may be better placed with *Balansiella* (a genus incorporated into *Claviceps* and not currently recognized). Whether the fungus is better placed with *Claviceps*, *Balansiella*, or elsewhere is at this point not clear, although the current arrangement, in which *Balansiella* is merged with *Claviceps*, assigns it to *Claviceps*. Therefore, it is retained as Möller (1901) originally intended, within *Claviceps*, but separate from *C. flavella*, until additional studies justify its placement elsewhere.

### 2.5. *C. bothriochloae* Tanda & Y. Murayama

*C. bothriochloae* was described by Tanda and Murayama (1992), based on a collection from *Bothriochloa parviflora* Ohwi from Japan. The sclerotia are 1.9–5.6 × 0.5–1.1 mm and dark brown or dark purple. Stipe and capitula are sulfur-colored. Capitula are 0.5–0.9 × 0.6–1.1 μm. Perithecia are 170–203 μm with ascospores of 71–104 μm. Conidia are elliptical or oval, 2.6–5.9 × 1.8–3.2 μm. In inoculation experiments no infections were seen on 19 species of cereals and grasses. Tests for ergot alkaloids were reported as obscure positive (Tanda and Murayama, 1992).

### 2.6. *C. cinerea* Griffiths

*C. cinerea* was first reported from *Hilaria* species in Arizona (Griffiths, 1901). Sclerotia are 1.5–3 cm × 1.75–2.5 mm, clavate, gradually tapering upward, dark gray at the base, fading to light gray to near white at the apex (Fig. 2a). Stipes are white. Capitula are subglobose, light gray, 1.75–2.75 mm in diameter. Perithecia are pyriform, 190–225 × 60–90 μm. Asci are 135–150 × 4–5 μm with ascospores 100–120 × 1–1.5 μm. Griffiths (1901) makes no mention of conidia in the technical description but provides a drawing showing small, roughly spherical conidia and larger conidia, which appear broadly fusoid and straight or slightly curved (Figs 1d and 1e). The two spore sizes could represent macro and micro conidia, although further studies are needed to understand conidiation in *C. cinerea*.

*C. cinerea* occurs on *Hilaria* and *Melica* species in the south central and south western United States. The host grasses are adapted to the dry plains and hills in the western United States (Hitchcock, 1971). The grasses have a short reproductive cycle; some plants can grow and produce mature seed within 2 months of the beginning of summer rains (Griffiths, 1901). Griffiths (1901)
determined that under moist soil conditions, sclerotia germinated and ascospores were produced within 1 month. This rapid germination of sclerotia coincides with the rapid reproductive cycle of the host grass. However, sufficient moisture is required for spore production, and in dry years rainfall may be sufficient for the host development but insufficient for sclerotial germination and production and release of ascospores (Griffiths, 1901; Sprague, 1950). In years of normal rainfall, release of spores coincides with the susceptible flowering period of the host plant.

**Figure 2** Variation in morphology of sclerotia among species of *Claviceps*. *C. cinereum* (a); *C. nigricans* (b); *C. paspali* (c); *C. purpurea* var. *purpurea* (d); *C. zizaneae* (e); and an unidentified species from *Diarrhena americana* (f).
2.7. *Claviceps citrina* Pažoutová, Fucikovsk, Leyna-Mir & Flieger

*C. citrina* occurs on *Distichlis spicata* (L.) Greene in the Texcoco region of central Mexico (Pažoutová et al., 1998). Sclerotia are brown to gray, 4–12 × 1–2 mm. Stipes are pale yellow with lemon yellow capitula (Fig. 3a). Capitula are 1–1.2 mm and papillate. Perithecia are 238–350 × 92–146 μm. Ascospores are 78–136 × 0.45–0.6 μm. Conidia are elliptical, 3.65–7.2 × 2.5–2.7 μm (Fig. 1f). The sclerotia contain no traces of clavines or lysergic acid or its peptide derivatives.

Sclerotia germinate rapidly, within 10–30 days after placement on moist sand. A cold conditioning period is not required for germination. The process from initial germination to mature ascospores is about 25 days. The Texcoco region is arid during the winter. Rapid germination coincides with a rapid development and seed set of the host plant during spring or fall rains (Pažoutová et al., 1998).

2.8. *C. cynodontis* Langdon

*C. cynodontis* was described by Langdon (1954a), based on collections from *Cynodon dactylon* (L.) Pers. from South Africa, Gold Coast, India, and Nyassaland. Sclerotia are deep brown, fusiform, straight or curved, up to 5 mm long. Stromata are lucid. Perithecia are 170 × 100 μm and asci are 90 μm long. Conidia are reniform, 10–20 × 4–6 μm. Few details of the species are provided in the technical description provided Langdon (1954a). *C. cynodontis* has a wide distribution, extending from India through Burma to the Philippine Islands, Africa, and Europe.

2.9. *C. cyperi* Loveless

*C. cyperi* was based on collections from *Cyperus* species collected in South Africa (Loveless, 1967). The descriptions were based on dried material at the National Herbarium of South Africa. The sclerotia are dark brown to almost black, up to 8 mm long by 1–1.5 mm wide. Dried stromata are pale straw in color. Capitula are subglobose, 1 mm in diameter and prominently papillate with a narrow collar at the base of the capitulum. Perithecia are 280–360 × 120–144 μm. Ascii are 90–120 × 2.5–3.5 μm with ascospores of 70–80 μm. Conidia are elliptical to oblong, 5.5–13 × 2–4 μm (Fig. 1g) (Loveless, 1967).

2.10. *Claviceps diadema* Diehl comb. nov.

In 1901, Möller described *Balansia diadema* from a *Panicum* species from Itajahy in Brazil. The fungus proliferates within a single flower or occasionally within and surrounding two adjoining flowers, forming a dense fungal mass with a dark yellow rind. Yellow stipes and capitula develop directly from the hypothallus while still attached to the plant (Fig. 3b). Stipes are 2–4 mm long and
capitula are 0.5–1 mm in diameter. Perithecia are 250 µm long with asci of 130 µm. In a nutrient medium ascospores germinate to produce oval, 7–9 µm-long conidia which, while still attached to the conidiophore, each germinate to produce a conidium, which germinates again in the same way, and so forth. Möller (1901) recognized the material as distinct from *Claviceps* by the lack of conidial fructification, the unusual germination pattern of the ascospores, and that the hypothallus encompasses the floral elements similar to *Balansia*, without producing a true sclerotium or requiring a resting period.

Henning (1904) believed there were sufficient differences between *Balansia diadema* and other *Balansia* species in terms of the sclerotium, conidial formation, etc., that it should be accommodated in a separate genus, *Balansiella* Henning. *Balansiella*, however, has been incorporated into the genus *Claviceps* and is not currently recognized. Based on Möller’s (1901) characterization of *B. diadema*, it appears to be more closely aligned with *Balansia* than *Claviceps*, and should be referred to as *B. diadema* Möller until further study justifies its placement elsewhere.

Diehl (1950) examined collections from *Ichanathus* species, which he believed to be conspecific with *B. diadema* Möller. The fungus from *Ichanathus* produced a sphacelium and was therefore a *Claviceps*. However, Möller (1901) clearly noted the absence of conidial fructification associated with the hypothallus in his description of *B. diadema* from a *Panicum* species. Diehl (1950) justified the synonymy on the assumption that Möller may have missed the conidia. This is not likely, since Möller specifically mentions looking for conidia both in situ and in culture. Given the detail found in his descriptions and technical drawings, I believe he would have seen conidia had they been there. Thus, the material examined by Diehl represents a species of *Claviceps* distinct from *B. diadema* Möll. and is therefore proposed as a new combination *C. diadema* Diehl. and *C. diadema* (Möller) Diehl is considered a synonym of *Balansia diadema* Möller.

Henning placed *C. pallida* [Wint] var. *orthocladae* P. Henn. in synonymy with *B. diadema* Möller. However, *C. pallida* var. *orthocladae* produces a sphacelium whereas *B. diadema* does not. Diehl (1950) also did not accept the synonymy of *C. pallida* var. *orthocladae* and *B. diadema* (see discussion under *C. orthocladae*).

2.11. *C. digitariae* Hansford

*C. digitariae* was originally described by Hansford (1940) from a collection on a *Digitaria* species from Uganda but was redescribed in greater detail by Herd and
Loveless (1965). Sclerotia are dark brown to almost black, oval to oblong, 1–4 mm × 1.5 mm. Stromata develop after 2–3 months under humid conditions, producing yellow to yellowish-white stipes with a tuft of white hyphae at the base. There is a collarlike appendage where the capitulum joins the stipe. Capitula are globose, yellow, 0.5–0.8 mm, with ostioles turning purple with age. Perithecia are ovate to broadly elliptic, 160 × 100–140 μm (Hansford, 1940) or 185–225 × 100–145 μm (Herd and Loveless 1965). Asci are 115–140 × 3–5 μm with ascospores of 90–120 × 1 μm. Conidia are predominantly elliptic, 10.5–17.5 × 3–5.5 μm (Fig. 1h).

Herd and Loveless (1965) note the smaller dimension of the perithecia reported by Hansford (1940), but believe they arrived at a higher value by taking only median sections through fully mature capitula. C. digitariae is known only from Digitaria species (Herd and Loveless, 1965).

2.12. C. fusiformis Loveless

C. fusiformis is important in millet production in the semiarid tropics of India and Africa. In India, C. fusiformis was believed to be C. microcephala (Shinde and Bhine, 1958) until correctly identified as C. fusiformis in 1973 (Siddiqui and Khan, 1973). Male sterile lines are the most susceptible to infection (Thakur et al., 1989). Epiphytotics occurred in India during the 1960s after the introduction of hybrids based on cytoplasmic sterility (Randhawa et al., 1997). The fungus is adapted to the warmer conditions of the tropics. In culture, fungal growth occurs at 20–35°C, with optimal growth at 27°C (Kumar and Thakur, 1995) and no measurable growth at 10°C (Roy and Kumar, 1989). The sclerotia require a storage temperature of 20–37°C before germination (Prakash et al., 1987). Prakash et al. (1987) concluded that high temperatures combined with periods of wetting and drying simulate the premonsoon conditions of semiarid tropics and would support good germination of sclerotia. Unlike C. purpurea var. purpurea, which requires cold temperatures to stimulate germination, Prakash et al. (1987) found that chilling of sclerotia reduced the percentage of germination of C. fusiformis. Disease incidence is favored at 20–30°C and high moisture conditions (Dakshinamoorthy and Sivapralasam, 1988; Thakur et al., 1991).

The sclerotia are light brown to blackish brown, pyriform to obpyriform, tapered at the apex, sometimes curved, 2–9 mm × 1–4 mm (Chahal et al., 1985; Loveless, 1967; Thakur et al., 1984). Sclerotia can germinate as early as 4 weeks and as late as 56 weeks at 25°C (Takur et al., 1984). At 28°C and continuous high moisture, sclerotia germinate over a 40–80-day period (Chahal et al., 1988). Up to 12–16 stromata can develop from a sclerotium (Chahal et al., 1988; Thakur et al., 1984). Stipes are pale purple or cream (Loveless, 1967; Thakur et al., 1984). Capitula are globose, 1–1.5 mm in diameter, grayish purple, slightly papillate (Loveless, 1967). Perithecia are ovate-pyriform, 130–175 × 60–95 μm.
Asci are 95–125 × 3–5 μm with ascospores slightly shorter than the asci (Loveless, 1967) or 103.2–176 × 0.4–0.5 μm (Thakur et al., 1984).

Macroconidia are fusiform 12–26.4 × 2.4–6 μm (Fig. 1i) (Thakur et al., 1984). Microconidia, produced in chains from germinated macroconidia, are globular, 2.4–10.8 × 1.2–4.8 μm (Fig. 1j) (Thakur et al., 1984). Budding can occur in microconidia (Chahal et al., 1985).

On glass slides, conidia germinate by 24 h at 15–35°C, with optimum at 25°C and no germination at 10 or 40°C (Mathur and Gopalan, 1988). Conidia on stigmas germinate within 12–16 h after inoculation (Willingale et al., 1986). Hyphae invade the stigmas within 24 h, reach the outer integuments within 30 h, and infect the base of the ovary within 36 h (Willingale et al., 1986). However, within 6 h of pollination stigmatic constriction begins and stigmas wither within 12 h, reducing the chance for ergot infection (Thakur and Williams, 1980; Willingale et al., 1986). Pollen germinates within 1 h and pollination within 16 h of infection can significantly reduce the level of ergot (Thakur and Williams, 1980).

Honeydew production occurs 4–6 days after inoculation (Willingale et al., 1986). Fresh honeydew collected from infected panicles and stored under laboratory conditions contained viable and infective conidia after 2 years, suggesting the potential for conidial carryover from one year to the next (Roy and Kumar, 1988). Macroconidia can germinate to produce conidiophores and microconidia in chains (Thakur et al., 1984). Sclerotia are visible 8–10 days and mature within 20–25 days after inoculation (Thakur et al., 1984). Alternate hosts for *C. fusiformis* include *Panicum antidotale* Retz. (Thakur and Kanwar, 1978) and *Setaria verticillata* (L.) Beauv. (Rathi and Panwar, 1993).

### 2.13. *C. gigantea* S. F. Fuentes, Ullstrup, & Rodriguez

*C. gigantea* was first described from Mexico, after infected corn was observed in the State of Michoacan during the 1960–1962 growing seasons (Fuentes et al., 1964). It is now considered endemic on corn in Mexico (Fucikovsky and Moreno, 1971). Mature sclerotia are large, often comma-shaped. The surface may be smooth or cracked, white to grayish brown, and the interior pale pink to lavender (Fuentes et al., 1964). Sclerotia germinate after 8 weeks under alternating weekly temperature/light periods of 22–28°C/diffuse light and 12°C/darkness, followed by 12°C for 4 months. Stipes are pink to reddish brown. Capitula are 0.32–0.55 cm in diameter with pink ostioles of perithecia protruding slightly. Perithecia are 338–444 × 152–164 μm and ascospores are 176–186 × 1.5 μm. Macroconidia are elliptical to spindle-shaped, 8.3–27.0 × 4.2–5.8 μm (Fig. 1k). Microconidia are ovoid, 4.2–6.7 × 2.5–3.3 μm (Fig. 1).
Conditions favorable for infection are 13 – 15°C and annual precipitation of more than 100 cm (Fucikovsky and Moreno, 1971). The sclerotia at first are white to cream-colored, soft, sticky, and hollow (Fuentes et al., 1964). Honeydew production is associated with the young sclerotia. As the sclerotia mature, internal cavities are replaced by pseudo-parenchymatous tissue, and the exterior becomes hard and horny.

2.14. **C. glabra** Langdon

*C. glabra* was described by Langdon (1942), based on a collection from *Digitaria longiflora* from Queensland, Australia. The sclerotia are black, subglobose. Stipes are pale straw yellow to cream-colored. Capitula are purple-red to chestnut-colored, 0.5 – 0.6 mm in diameter. Perithecia are 165 – 180 × 115 – 130 μm and ascospores are 70 – 110 μm. Conidia are elliptical or curved, 12.5 – 20.5 × 4.0 – 7.0 μm. Characteristic of this species is the lack of loose hyphae that normally develop around the young stroma. A species of *Claviceps* described as *C. amamiensis* from *Digitaria ciliaris* from Japan is very similar in description to *C. glabra* (see Sec. 2.2, *C. amamiensis*).

2.15. **C. grohii** J. W. Groves

A *Claviceps* on *Carex* species was first reported from Canada in 1911 (Groh, 1911), but a formal description was not initiated until 1939 (Groves, 1943). Sclerotia are blackish violet to purplish black, 5 – 15 × 1 – 3 mm, semicylindrical, flattened on one side, straight or curved. Sclerotia germinated after incubation in moist sand at 0°C for 3 months followed by placement in a greenhouse at 4 – 15°C. Germination began after 6 weeks in the greenhouse, but mature ascospores did not develop until about 10 weeks. Stipes are blackish violet to blackish brown with a tuft of violet mycelium at base. Capitula are 0.8 – 2.0 mm in diameter, pink buff to orange vinaceous with darker orange perithecial ostioles. Perithecia are ovoid, 150 – 300 × 100 – 150 μm. Asci are 100 – 175 × 5 – 6 μm. The dynamics of infection and whether a sphaecelium develops needs to be determined. Conidia are slightly arcuate with rounded ends, 10 – 16 × 3 – 5 μm (Fig. 1m) (Langdon, 1952a).

2.16. **C. hirtella** Langdon

*Claviceps hirtella* was described by Langdon (1942), based on a collection from *Eriochloae pseudoacrotricha* ( Stapf ex Thellung) C. E. Hubbard ex S. T. Blake from Queensland, Australia. Sclerotia are subglobose, yellowish to deep brown. Stipes emerge through a mass of loose hyphae, which persists as a ring of white mycelium at the base of the stipe. The name of this *Claviceps* is based on the shaggy appearance of the developing stroma. Stipes are grayish pink and glabrous. Capitula

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are chestnut brown to brown, 0.6–0.9 mm in diameter. Perithecia are 185–215 × 130–165 μm with ascospores of 70–85 μm. Conidia are curved to elliptical, 11.0–16.5 × 4.5–6.5 μm. Hosts include species of *Eriochloa*, *Brachiaria*, and *Paspalidium*. *C. hirtella* is known only from Australia.

2.17. **C. imperatae** Tanda & Kawat.

*C. imperatae* was described by Tanda and Kawatani (1976) based on a collection from *Imperatae cylindrica* Beauv. var *koenigii* (Durand and Schinz) from Japan. Sclerotia are purplish brown or dark brown, cylindrical, ovoid or subglobose, straight or curved, 1.2–10.5 mm long. Stipes are brownish purple at the base and reddish gray at the apex, with the occurrence of purplish hyphae at the base of the stipe. Capitula are maroon or reddish brown, 0.7–1.4 × 1.0–1.5 mm with perithecia, 134–189 × 67–122 μm. Ascospores are 32–63 × 0.4–0.7 μm. Conidia are elliptical or oblong, 6.7–27.5 × 3.7–9.2 μm. Alkaloids include agroclavine, ergometrine, ergotamine, ergocormine, and ergocristine.

2.18. **C. inconspicua** Langdon

*C. inconspicua* is known from *Hyparrhenia filipendula* (Hochst.) Stapf from Australia (Langdon, 1950b). The name is derived from the small, dark brown to black, cylindrical or fusoid, 2–5 mm-long sclerotia which are inconspicuous within the spiklets. Sclerotia germinate to produce one to many stromata. Stipes are anthracene purple and capitula are raisin black, 0.3–0.6 mm in diameter. There is a ring of whitish hyphae at the base of the capitulum. Perithecia are 155–180 × 105–125 μm with asci of 140–175 × 4 μm. Conidia are 15–20 × 5–10 μm with sides straight or slightly curved and both ends rounded.

Langdon (1950b) cited the occurrence of ergot on *Hyparrhenia* species in Brazil, Southern Rhodesia, Kenya, and Sierra Leone, based on occurrence of the saprophytic fungus *Cerebella* on the honeydew stage. Langdon (1950b) believed that *C. inconspicua* was native to Australia and likely not an introduced species. However, Langdon (1952a) raises the possibility that *C. inconspicua* could be an Australian variant of the widely distributed *C. pussila*.

2.19. **C. lutea** Möller (= *Ustilagopsis compactiuscula* Speg.)

Möller (1901) described *Claviceps lutea* from a *Paspalum* species from Brazil (Möller, 1901). Langdon (1952a) examined the figures published by Möller (1901) and examined collections of *C. lutea* from Brazil and concluded that what Möller believed to be a *Paspalum* was most likely a *Panicum*. Thus the host of *C. lutea*, as provided by Möller (1901), should be amended to refer to *Pancium* rather than *Paspalum*.
In *C. lutea*, a loose mycelium and associated conidial production develops within and surrounding the flower. Conidia are $9 \times 2 \mu m$. Sclerotium formation, however, occurs above the spikelet and takes the form of a caplike, curved bulge, up to 3 mm in diameter. The rind is a pronounced yellow color and slightly grainy. A firm interwoven mycelium connects the sclerotium to the ovary and remains attached as a conical appendage after maturity and separation from the flower. When Möller (1901) placed sclerotia on moist sand in May, they germinated by December. Stipes are bright yellow. Capitula are honey yellow, round, 1.5 mm in diameter, and are similar in appearance to *C. balansoides*. Asci are 250 μm with ascospores of 180 μm. Möller (1901) also observed conidial production in culture.

### 2.20. *C. maximensis* Theis

*C. maximensis* was first described from *Panicum maximum* Jacq var. common guinea from Puerto Rico, where the forage grass was found frequently infected (Thies, 1952). Sclerotia are 2–9 mm × 1 mm, straight or slightly curved, tapered toward the apex, brown to gray-brown. Stipes are pale green, becoming yellow as the capitulum matures. Capitula are subglobose, light yellow to brown, 0.7–1.0 mm in diameter. Perithecia are flask-shaped, 240–280 × 120–160 μm. Asci are 2.5–3.0 × 105–139 μm with ascospores of 0.5 × 95–126 μm. Conidia are elliptical, 10–30 × 3.5–11 μm. Tests for alkaloids were negative.

Sclerotia germinate in approximately 20 days (Thies, 1952). Guinea grass is drought-tolerant and well adapted to arid conditions (Thies, 1952). Loveless (1964) reported that *P. maximum* is indigenous to Africa and was likely introduced to Puerto Rico from Africa, along with *C. maximensis*, which is also believed to be indigenous to Africa. In Rhodesia, the sclerotia were found to survive the dry season from May through November and to germinate during the rainy season in December–January (Loveless, 1964a).

### 2.21. *C. microspora* Tanda (= *C. microspora* var. *kawatanii* Tanda)

Tanda (1985) described *C. microspora* from *Arundinella hirta* (Thunb.) C. Tanaka from Japan. Sclerotia are cylindrical, taper at each end, blackish purple or black. Stipes are dark purple or light black with no tuft of mycelium at the base. Capitula are globose or slightly depressed globose, dark purple or light black, 0.3–0.7 × 0.3–1.1 mm, with protruding dark purple perithecial ostioles. Perithecia are oblong or ovoid, 147–214 × 63–123 μm. Asci are 95–161 × 2.1–3.9 μm with ascospores of 88–133 × 1 μm. Conidia are ellipsoid or subglobose, 2.2–4.9 × 1.3–3.2 μm (Fig. 10). Alkaloids were not detected. Isolates of *C. microspora* did not infect cereal and grass hosts commonly infected by *C. purpurea* var. *purpurea*.
A *Claviceps* on *Ecciloopus cotulifer* A. Camus, previously listed as *C. purpurea*, var. *purpurea* was described by Tanda as a new variety, *kawatani*, of *C. microspora* (Tanda, 1991). Sclerotia are black to dark brown, obclavate, straight or slightly curved, 4.1–9.5 × 0.9–2.6 mm. Sclerotia placed outside in February germinated by August. Stipes are light purplish brown with no tuft of mycelium at the base. Capitulum are dark purple, 1.2 × 1.4 mm. Perithecia are 186–242 × 81–142 μm. Asci are 125–231 μm with ascospores 84–179 μm. Conidia are subglobose, 2.5–4.0 × 1.9–3.2 μm. Alkaloids were not detected. Isolates *C. microspora* var *kawatani* did not infect cereal and grass hosts commonly infected by *C. purpurea* var *purpurea* (Tanda, 1991). Tanda (1991) separated *C. microspora* var *kawatani* from *C. microspora* based on somewhat larger perithecia, asci, and ascospores. Variability in size of perithecia, asci, and ascospores is well known in *Claviceps*, and such measurements alone cannot serve as the basis for establishment of varieties.

### 2.22. *C. nigricans* Tul.

*C. nigricans* is morphologically very similar to *C. purpurea* var *purpurea* but differs in host range. Sclerotia are black, semicylindrical, elongated, often curved (Fig. 2b) (Longdon, 1952a). Hosts for *C. nigricans* include species of *Eleocharis* and *Scirpus*, members of the Cyperaceae. *E. palustris* is widely distributed and occurs in temperate climates in North America, Europe, Asia, and Northern Africa (Wiersema and Leon, 1999). The stipes are bluish violet at first, later dark violet (Ellis and Ellis, 1997; Tulasne, 1853). Capitula are 1.25–2 mm in diameter, initially cinnamon-buff, turning dark violet to blackish violet, with perithecia markedly protuberant (Ellis and Ellis, 1997; Langdon, 1952a; Tulasne, 1853). Ascospores are 89–90 × 1 μm (Ellis and Ellis, 1997). Conidia are oblong, 8–12 × 3–4 μm (Fig. 1p) (Langdon, 1952a).

### 2.23. *C. Orthocladae* (Henn.) Diehl (= *Balansiella orthocladae* Henn.; = *C. pallida* Wint. var *orthocladae* Henn.)

*C. orthocladae* was first described by Henning (1900) from an *Orthocladae* species from St. Catharine, Brazil. A sclerotia-like mycelium develops during parasitism of the ovary and appears subglobose, spirally compressed, and yellow. Stromata are stipitate, with stipes and capitula yellow. Capitula are 1–2 mm in diameter, hemispherical with prominent ostioles (Fig. 3c). Perithecia are oblong-ovoid. Asci are 150–180 × 3 μm with ascospores 0.5 μm wide. Diehl (1950) observed the sphacelial stage and transferred Henning’s *Balansiella orthocladae* to *Claviceps*.
2.24. *C. panicodearum* Tanda & Harada (= *C. miscanthi* Sawada)

*C. panicodearum* was described from *Isachne globosa* O. Kuntze in Japan (Tanda and Harada, 1989). Sclerotia are dark purple or black, oblong, ovoid or obclavate, somewhat flattened and usually straight, 1.7–6.6 × 0.8–2.5 mm. Stipes are blackish purple with a tuft of mycelium at the base. Capitula are subglobose or depressed globose, darker than the stipe in color, with dark purple perithecial ostioles, 0.3–1.1 × 0.3–1.6 mm. Perithecia are oblong or ovoid, 219–312 × 122–194 µm. Asci are 126–214 × 2.5–3.5 µm with ascospores 95–193 µm. Conidia are ellipsoid or ovoid, 2.5–5.9 × 1.6–2.5 µm (Fig. 1q).

Sclerotia planted outside in February began germinating by early June, and stromata matured by late June (Tanda and Harada, 1989). *C. panicodearum* is also known to occur on *Miscanthus* species (Tanda 1991). In a comprehensive examination of *Claviceps* on *Miscanthus* species in eastern Asia, Tanda (1991) identified the *Claviceps* infecting *Miscanthus* spp. as *C. panicodearum*.

2.25. *C. paspali* F. Stevens & J.G. Hall (= *C. deliquescens* Speg.; = *C. rolfsii* F. Stevens & J. G. Hall)

*C. paspali* was first described on *Paspalum dilatatum* Poir. and *P. laeve* Michx. in the southeastern United States (Stevens and Hall, 1910). The sclerotia are globose, 2–4 mm in diameter, irregularly roughened on the surface, and yellowish gray in color (Fig. 2c) (Brown, 1916; Langdon, 1952a). As sclerotia mature, three layers develop: an outer layer composed of dead cells; an inner layer of densely compacted and interwoven cells, which includes 90% of the sclerotium; and a transitional layer, intermediate in appearance between the outer and inner layers (Stewart, 1957). The stipe originates from the innermost layer (Stewart, 1957).

Sclerotia overwintered outdoors germinate within 1–2 weeks after placement in moist sand, with ascospore production in 4 weeks (Luttrell, 1977). A cold conditioning period at 5–10°C for longer than 6 weeks is required for germination of sclerotia (Cunfer and Marshall, 1977; Luttrell, 1977), although a low percentage of sclerotia germinate without a cold period (Cunfer and Marshall, 1977). After cold conditioning, sclerotia germinate after 13–101 days, most after 20–50 days (Cunfer and Marshall, 1977). Rainy conditions favor sclerotal germination (Brown, 1916), and germination occurs in the spring at about the same time *Paspalum* species flower (Cunfer and Seckinger, 1977). Sclerotia germinate to produce one to several stromata (Brown, 1916). Stipes are whitish. Capitula are whitish at first, then bright yellow, and finally brownish (Brown, 1916). Perithecia are oval, 175–350 × 8–120 µm (Langdon, 1952a; Stevens and Hall, 1910). Asci are 150–170 µm with ascospores of 70–100 µm (Brown, 1916). Germinated sclerotia can tolerate cycles of wetting and drying.
When immature fruiting bodies are removed, the sclerotium develops new ones (Stewart, 1957).

Male sterile plants are more susceptible than male fertile plants of *Paspalum notatum* Fluegge (Burton and Lefebvre, 1948). Fertilized florets of *Paspalum* species are resistant to *C. paspali*, and varieties with high seed set have less ergot (Stewart, 1958).

Infection is noticeable within 7 days of inoculation with ascospores (Brown, 1916). The honeydew stage lasts a few days, and sclerotia are noticeable a week after the honeydew stage (Brown, 1916).

The process of infection was described by Luttrell (1977). Conidial germ tubes penetrate between cells of the stigma and style and grow downward into the style to the ovary. Invading hyphae enter the apex of the ovary through the styles and penetrate the inner layers of the ovary. After 2 days an intercellular mycelium occupies the ovary wall and ovule. A white mycelium develops on the surface of the ovary within 3 days, and by the fourth day a massive stroma surround the ovary, although the receptacle remains uninfected. Honeydew appears 4–7 days after inoculation, and continues for about 5 days. Conidia an oblong, 8–16 × 4–7 µm (Fig. 1r) (Brown, 1916; Langdon, 1952a). Sclerotia emerge 9–11 days after inoculation and are mature after 4 weeks. Luttrell (1977) noted that conidia germinated to produce secondary conidia, although the role of secondary conidia as infective airborne inoculum, as in the case of *C. africana*, has not been established.

*Paspalum dilatatum* was introduced into the southern United States from Uruguay or Argentina about the mid-1800s and is now common through the Gulf states (Hitchcock, 1971). It is not established if *Paspalum* in the United States was originally infected by local strains of *C. paspali* from native species of *Paspalum* or if the fungus was introduced from South America with *Paspalum* seed. However, *C. paspali* was introduced into Australia, likely with seed, during the mid-1930s. The exact timing of the introduction is not clear. Langdon (1963) believed that reports of *C. paspali* in Australia prior to 1935 were in error (Langdon, 1963). Early reports of ergot on *Paspalum scrobiculatum* L. (= *P. orbiculare* G. Forst.) were from *C. queenslandica*, with no confirmed reports of *C. paspali* on *P. dilatatum* in Australia prior to 1935 (Langdon, 1963). *C. paspali* has followed the introduction of *Paspalum* species into numerous other countries (Langdon, 1952a).

*C. rolfsii* was distinguished from *C. paspali* based on fewer perithecia within the capitulum, larger perithecia (816–225 µm), larger asci (375 × 3 µm), and longer ascospores (160–275 × 0.5 µm). Wolf and Wolf (1947) collected and germinated sclerotia from *Paspalum laeve*, *P. dilatatum*, and *P. floridanum* Michx. over several years. Size of sclerotia was proportional to the size of the host seed, but perithecia, asci, and ascospores all corresponded with measurements for *C. paspali*. *C. rolfsii* is listed as a separate species by
Skalicky and Stary (1962) and synonymous with *C. paspali* by Langdon (1954). Langdon (1952a) believed it likely that Stevens and Hall (1910) based their description of *C. rolfsii* on an abnormal form of *C. paspali*.

### 2.26. *C. phalaridis* J. Walker

*C. phalaridis* is known from New South Wales and Victoria, Australia. It was first described from *Phalaris aquatica* L. (= *P. tuberosa* L.) (Walker, 1957). Sclerotia are fawn-colored, spherical to oblong, 1.5–2.5 × 1–2 mm. Stipes are initially white, becoming pale purple drab to dark purple drab. The capitulum is dark purple to black, globose, 0.6–1.4 mm in diameter. Perithecia are pyriform, 240–320 × 70–120 μm. Asci are 130–270 × 4 μm, with ascospores of 125–240 μm. Conidia are oblong to cylindrical, straight or slightly curved, tapering slightly, 5.5–15 × 2–4 μm. Low temperature are not required for germination, but a resting period under dry conditions of about 5 months at room temperature is needed to break dormancy.

Walker (1970) extended the host range to include *Lolium rigidum* Gaudin, *Vulpia bromoides* (L.) S. F. Gray, *Dactylis glomerata* L., and *Danthonia* species. It is important to note that *C. phalaridis* differs from all other species of *Claviceps* in developing systemically within the host plant (Walker, 1970). At flowering, the mycelium envelopces the anthers and ovary, which are incorporated into the mature sclerotium. Diseased inflorescences have 100% infected florets. Uecker (1980) reported that each cell of the ascospore can produce a single septate conidiophore and conidium, and can do so while still in the ascus.

The systemic nature of *C. phalaridis* is not typical of *Claviceps*. Walker (1970) and Uecker (1980) suggested *C. phalaridis* should be considered a *Balansia*, although the systemic nature of infection would be more characteristic of *Epichloë* than *Balansia*. However, phylogenetic analysis of 5.8S rDNA and ITS1 and ITS2 spacers by Pazoutová (2001) clearly place it within *Claviceps*.

### 2.27. *C. platytricha* Langdon

*C. platytricha* was described by Langdon (1942), based on a collection from *Ischaemum australe* R. Br. from Queensland, Australia. Sclerotia are cylindrical, straight or curved, and dark chestnut in color. Stipes are umber-colored, glabrous, except for loose hyphae at the base. Capitula are globose, 0.6–1.2 mm in diameter and drab-colored. Perithecia are pyriform 180–215 × 110–150 μm. Asci are 65–105 × 4 μm, with ascospores up to 105 μm. Conidia are 7.5–11 × 3.5–5.5 μm with parallel sides, rounded ends, rarely curved. *C. platytricha* is known only from *I. australe* in Australia, but the occurrence of ergot on *Ischaemum* species in Asia may also be due to this species (Langdon, 1952a).
2.28.  *C. purpurea* (Fr.: Fr.) Tul. var *purpurea* (= *C. litoralis* Kawat.; = *C. microcephala* (Wallr.) Tul.; = *C. sesleriae* Stäger; = *C. setulosa* (Quel.) Sacc.; = *Cordyceps setulosa* Quel.)

The best known of the *Claviceps* species is *C. purpurea* var *purpurea*, easily recognized by elongated, purple-black sclerotia that extend from infected florets. The species has been extensively studied and the focus of several comprehensive literature reviews (Barger, 1931; Bove, 1970; Kren and Cvak, 1999). *C. purpurea* var *purpurea* is common in temperate climates and occurs on some 300–400 susceptible hosts (Brady, 1962; Kawatani, 1953), including the economically important cereal grains and forage grasses. Nearly all hosts of *C. purpurea* var *purpurea* occur in the Pooidae.

Sclerotia are purplish black, cylindrical to fusoid, with rounded or tapered apex, straight or curved, and variable in length, 2–25 mm (Fig. 2d). In general, sclerotia are one to several times larger than the host seed. Sclerotia of *C. purpurea* var *purpurea* must be exposed to a period of cold temperature (0–10°C) before germination will occur (Kirchoff, 1929; Mitchell and Cooke, 1968). Sclerotia germinate in the spring, about the time that grasses begin to flower. Once the cold conditioning requirement has been met, germination of sclerotia depends on moisture and temperature conditions. Optimal temperature for germination is 10–25°C (Kirchoff, 1929; Mitchell and Cooke, 1968) but is reduced above 25°C (Mitchell and Cooke, 1968). Larger sclerotia have a greater capacity for production of stromata than smaller sclerotia (Cooke and Mitchell, 1967), and up to 60 stromata may develop from a sclerotium (Sprague, 1950). Saturated relative humidity favors elongation of the stipe (Hadley, 1968). Stipes are pale yellowish at first, later purple-violet or lilac with tuft of hyphae at base (Atanasoff, 1920; Tulasne, 1853). Capitula are globose, about 2 mm across, at first light yellowish, later reddish-flesh colored to pale fawn or cream (Atanasoff, 1920; Langdon, 1952a; Tulasne, 1853), and each contain about 200 perithecia arranged over the outer surface (Fig. 3d) (Cooke and Mitchell, 1966). A collar is at the base of the capitulum (Atanasoff, 1920). Perithecia are flask-shaped, 100–150 × 160–200 µm (Tulasne, 1853) to 150–175 × 200–250 µm (Sprague, 1950) to 100–170 × 150–300 (Langdon, 1952a). Ascospore lengths have been reported as 50–76 µm (Atanasoff, 1920), 100 µm (Dennis, 1968), 100–110 µm (Loveless and Peach, 1986), 120 µm (Tulasne, 1853), and up to 140 µm (Langdon, 1952a).

Spore-trapping studies confirm the release of ascospores in the spring, about the time of flowering in grasses (Alderman, 1993; Mantle and Shaw, 1976; Wood and Coley-Smith, 1982). Wood and Coley-Smith (1982) found that sclerotia collected from one source germinated over a period of 6 weeks, but those from many sources germinated over a period of 5 months. Rainfall or high soil moisture content is required to release ascospores (Alderman, 1993; Mantle
and Shaw, 1976). Ascospore germination and subsequent infection of the stigma, style, or ovary wall occurs within 24 h (Shaw and Mantle, 1980a; Luttrell, 1980; Campbell, 1958). However, resistance to infection can occur after pollination (Campbell and Tyner, 1959; Cunfer et al., 1975; Puranik and Mathre, 1971; Watkins and Littlefield, 1976; Darlington and Mathre, 1976). Colonization of the ovary proceeds from the base of the ovary upward but does not grow below into the rachilla (Luttrell, 1980; Campbell, 1958; Shaw and Mantle, 1980a). Within 5 days a hymenium is produced, and within 7–10 days a sphaelium develops (Campbell, 1958; Rapilly, 1968; Wood and Coley-Smith, 1982). Conidiogenesis is phialidic (Rykard et al., 1984). Conidia are elliptical, 4–6 × 2–3 μm (Tulasne, 1853) to 4–14 × 3–7 (Fig. 1s) (Langdon, 1952a), although conidal size and shape can vary somewhat with host (Loveless and Peach, 1974) or environment (Pazoutová et al., 2000b). Conidial size may also vary depending on the osmotic value of the honeydew, with larger conidia produced at higher water potentials (Kirchoff, 1929). The water potential of honeydew, lowered by the high concentration of sugars, prevents conidial germination in situ (Cunfer, 1976). Conidial germination is optimal at 19–20°C (Rapilly, 1968). In the British Isles, C. purpurea var purpurea has been found to overwinter on Poa annua L., with successive infections occurring during mild periods throughout the winter (Vizoso et al., 1984).

Stäger (1907) differentiated C. sesleriae from C. purpurea var purpurea based on larger conidia (10.5–14 × 3.5–0.7 μm) and a narrower host range. C. sesleriae is considered synonymous with C. purpurea var purpurea (Bove, 1970; Langdon, 1954; Skalicky and Stary, 1962).

C. litteralis is known from Leymus mollus (Trin.) Hara (= Elymus mollis Trin.) in Japan (Kawatani, 1946). Sclerotia are narrowly cylindric, straight or somewhat curved, pale yellow-brown when immature and black, brown-purple to dark brown when mature, 3.5–28 × 1.2–6 mm wide. Stipes are pale flesh-colored to pale red-brown. Capitula are spherical or subglobose, brown-purple to dark purple, 0.6–2 mm high × 0.8–2.5 mm wide. Perithecia are pyriform to oblong obovate, 135–250 × 75–150 μm. Asci are 75–160 × 2.2–4.2 μm, with ascospores of 65–140 × 0.4–1.2 μm. Conidia are ovate-elliptical, 3.1–18.5 × 2.3–7.1 μm. Skalicky and Stary (1962) listed C. litoralis as synonymous with C. purpurea. Elymus species are well-established hosts for C. purpurea var purpurea, and the description for C. litoralis is consistent with that of C. purpurea var purpurea.

C. microcephala is generally considered synonymous with C. purpurea var purpurea (Langdon 1954b; Skalicky and Stary, 1962). However, Ellis and Ellis (1997) list C. microcephala as a separate species characterized by small sclerotia that germinate to produce white capitula, about 1 mm in diameter, on crimson stalks.

Quelet (1964) described Cordyceps setulosa from Poa growing on the mountainsides of the Jura and Vosges. It was listed as Claviceps by Saccardo (1883). The capitulum is globose, 1 mm in diameter, globose, fawn-colored,
papillate with brown ostioles. A tuft of hyphae is at the base of the capitulum. It was listed in synonymy with *C. purpurea* var. *purpurea* by Langdon (1952a, 1954b) and by Skalicky and Stary (1962). The description of *C. setulosa* by Quelet (1964) is consistent with that for *C. purpurea* var. *purpurea*.

2.29. **Varieties of *C. purpurea***

Stüger (1903, 1907, 1910, 1922) and Tanda (1979, 1980) defined varieties of *C. purpurea*, based largely on host range. Such varieties have not been widely accepted due to conflicting results among host range studies and variability among strains of *C. purpurea*.

2.30. ***C. purpurea* var. *spartinae*** R. Duncan, J. White, R. Sullivan, S. Alderman, and J. Spatafora

*Spartina* species are well adapted to coastal estuaries, where they often dominate. They are important soil binders in coastal and interior marshes and can facilitate land reclamation (Hitchcock, 1971). In the United States, important species include *S. alterniflora* Loisel. and *S. patens* (Ait.) Muhl. In Great Britain, *S. angelica* C. E. Hubbard has come to dominate a narrow niche in tidal estuaries, forming dense monospecific stands (Gray et al., 1990a). It has been planted in temperature zones throughout the world for stabilization of tidal mud flats (Gray et al., 1990a).

Loveless (1971) recognized that conidia of *Claviceps* from *Spartina x townsendii* H. and J. G. Grov differed in size and morphology from *C. purpurea* var. *purpurea* and suggested that it could differ taxonomically. In infection studies, Mantle and Shaw (1977) were not able to infect male sterile wheat with a strain from *S. x townsendii*. In inoculation experiments using a strain of *C. purpurea* var. *purpurea* from rye, Mantle (1969) observed that only two small (4-mm) sclerotia developed on *Spartina*, indicating that although infection may be possible, the resulting infections were not typical. Pazoutová et al. (2000b) recognized *Claviceps* from *Spartina* as a chemorace, based on a unique RAPD pattern. Samuelson and Gjerstad (1966) reported that the alkaloid profile for *Claviceps* from *Spartina* differed from that of *C. purpurea* var. *purpurea*. Duncan et al. (2002) formally described the ergot from *Spartina* as *C. purpurea* var. *spartinae* based on morphology and phylogenetic analysis of the ITS1 region. Sclerotia are 17.2 ± 3.92 × 1.7 ± 0.5 mm, purple-brown or dark brown. Stipes are purple-brown. Capitula are 1.3 ± 0.4 mm, globose to subglobose, punctate with reddish ostioles. Perithecia are ovoid, 144.2 ± 23.9 × 91.8 ± 11.2 μm. Asci 62.5 ± 0.9 × 2.6 ± 0 μm with asci 84.4 ± 4.5 × 8 ± 0 μm. Conidia are cylindrical, 8.06 ± 1.8 × 4.12 ± 1.3 μm.

The first observation of *Claviceps* on *Spartina* in the United States was by Tracy and Earle (1895) in Mississippi in 1892. Eluterius (1970) surveyed...
Spartina along the Gulf Coast for ergot in 1968 and found 96.5% of mature S. alterniflora culms infected, with 71% of seed on infected panicles replaced by sclerotia, resulting in 68.5% reduction in seed production. In North Carolina, Gessner (1978) examined S. alterniflora at 11 marsh areas in Carteret County and found Claviceps at all sites. Sclerotia and inflorescences infected per square meter ranged from 3 to 183 and from 2 to 47, respectively (0.2–1% infected seed) (Gessner, 1978).

In Great Britain, evidence of ergot infection was first observed in 1960–1961, although infections were rare and resulted in only small 4-mm) immature sclerotia (Boyle, 1976). The small sclerotia were similar to those described by Mantle and Shaw (1976) when Spartina was inoculated with a strain of C. purpurea var. purpurea from rye. It is likely that these infections were from C. purpurea var. purpurea. Aside from this rare occurrence, ergot was not detected on Spartina from 1958 to 1974 (Boyle, 1976). However, in 1975 an epiphytotic of ergot suddenly appeared (Boyle, 1976). Conidia averaged 9.8 × 3.8 μm from S. x townsendii, 10.2 × 3.9 μm for S. anglica (Boyle, 1976), consistent with those for C. purpurea var. spartinae. At Poole Harbour in 1985, 16% of all spiklets were infected (Gray et al., 1990b). In 1988, a mean of 85.2% of all inflorescences contained at least one ergot (Gray et al., 1990b). It appears that C. purpurea var. spartinae may have been introduced into Great Britain during the early 1970s.

Eleuterius and Meyers (1974) found that ergot severity was 7–10 times greater on plants which had colonized barren spoil areas or man-made beaches, where up to 100% of panicles can be infected and up to 95% of seed replaced by sclerotia (Eleuterius and Meyers, 1974). In undisturbed marshlands, 10% incidence with 1% seed replaced was observed (Eleuterius and Meyers, 1974). Eleuterius and Meyers (1974) provided evidence that greater exposure, aeration, and periodic drying of the substrate due to increased elevation favored germination of sclerotia and dissemination of ascospores. They determined that sclerotia stored dry for 6–9 months germinated readily, but that sclerotia stored wet in brackish water failed to germinate. Sclerotia germinated under total darkness, but no capitulum was formed unless the stroma was exposed at least periodically to light. Stromata under lower light developed longer stipes than those exposed to higher light intensities (Eleuterius and Meyers, 1974). Thus, sclerotia covered with mud would germinate, but the capitulum would not mature until it reached the surface, and periodic drying would be required for the airborne release of ascospores.

Secondary disease spread of C. purpurea var. spartinae by conidia may be responsible for high levels of infection. In the United States there is a progression of maturity of several species of Spartina from southern to northern states along the eastern coast, where earlier-infected plants can be a source of inoculum.
In Great Britain, *Spartina* species flowers between July and November, creating a long window of opportunity for disease spread by conidia (Gray et al., 1990b).

### 2.31. *C. pussila* Cesati

The original description of *C. pussila* by Cesati dealt only with characteristics of the stromata, prompting Langdon (1950a) to redescribe the species. Loveless (1964a) verified the description of Langdon based on collections from Rhodesia and found them to be similar except for a slightly greater range in the size of conidia. Sclerotia of *C. pussila* are brown to black, and cylindrical with tapered ends. Stipes are pale yellow in color, with tufts of white hyphae persisting at the base. Capitula are globose, 0.5–1.0 mm, and dark straw in color. A collar-like appendage surrounds the base of the capitulum. Perithecia are subglobose, 220–300 × 125–165 μm. Ascii are 55–160 μm. Conidia are mostly triangular, some elliptic, 10–15.5 × 5.0–7.5 μm (Fig. 1) (Langdon, 1950a). The triangular conidia are characteristic of the species.

*C. pussila* has a wide host range, primarily on hosts in the Andropogoneae, including species of *Bothriochloa*, *Dicanthium*, *Capillipedium*, *Themeda*, *Cymbopogon*, *Heteropogon*, and *Vetiveria* (Langdon, 1950a), and can be found in warm temperate climates in Africa, Australia, Asia, and Europe (Brady, 1962; Langdon, 1952b; Loveless, 1964a). Reports of *C. pusilla* in N. America were challenged by Langdon (1952a), based on examination of collections from *Bothriochloa saccharoides* (SW.) Rydb. (= *Andropogon saccharoides* SW.) and *Andropogon geradii* Vitman (= *A. furcatus* Muhl. ex Willd.). The occurrence of *C. pusilla* in North America has not been clearly established.

Langdon (1942) reported that ascus length varied from 55 to 70 μm in immature capitula, from 75 to 60 μm in mature capitula, and from 115 to 150 μm in very old capitula. The sclerotia do not require cold conditioning for germination (Langdon, 1950a). Sclerotia can survive longer than 1 year if stored dry (Langdon, 1950a).

### 2.32. *C. queenslandica* Langdon

*C. queenslandica* is known only from *Paspalum orbiculare* in Australia (Langdon, 1954a). Sclerotia are subglobose, up to 3 mm wide, yellow with a matte surface. Capitula are globose, 0.75–1.5 mm, and yellow, with perithecia of 220–260 × 120–150 μm. Ascii are 80–170 × 5 μm with ascospores of 70–130 μm. Conidia are oblong, or approaching wedge-shaped, 10–20 × 3.5–5 μm. In infection studies, Langdon (1954a) determined that *P. orbiculare* can be infected by both *C. paspali* and *C. queenslandica*, but that the *P. paspali*, the typical host for *C. paspali*, is not infected by *C. queenslandica*.
2.33. **C. ranunculoides** Möller

*C. ranunculoides* was described from a *Setaria* species, collected near Blumenau, Brazil (Möller, 1901). The sclerotia are blue-black, bent and hornlike. Under-developed sclerotia are associated with an orange-colored sphacelium producing oval conidia, 7–8 × 3 μm. Sclerotia collected in May and placed on moist sand germinated in January, producing bright yellow capitula. Perithecia are 400–500 μm long and are arranged in what looks somewhat like a bouquet of perithecia, much like the head of *Ranunculus*. Asci are 300 × 4 μm with a very flat cap. Ascospores are 160 μm long and, when placed in water or nutrient solution, break into some 30, approximately 5-μm-long, cells. In culture, conidia are 8–12 × 2 μm.

2.34. **C. rhynchelytri** G. W. Herd and A. R. Loveless

*C. rhynchelytri* was described from *Rhynchelytrum repens* (Willd.) C. E. Hubbard collated at Salisbury, Rhodesia (Herd and Loveless, 1965). Sclerotia are dark brown, ellipsoidal, oval in cross section, 5 × 1 mm. Sclerotial germination begins within 4–6 days, and stromata mature within 14 days after germination. Stipes are straw-colored. Capitula are subglobose or hemispherical, up to 1.1 mm in diameter, and bright yellow in color. Perithecia are ovoid, 185–205 × 100–125 μm. Asci are 140–145 × 1 μm, with ascospores of 105–150 × 1 μm. Conidia are hyaline and reniform, 5.5–10.5 × 2.5 μm (Fig. 1u). *C. rhynchelytri* is believed to be widespread in southern Africa (Herd and Loveless, 1965).

2.35. **C. sorghi** Patil Kulkarni, Seshadri & Hegde

The Sphacelia stage of *Claviceps* on sorghum was first detected in India in 1917 (McRae, 1917). The species is restricted largely to the Indian subcontinent, where it has been a problem in hybrid sorghum production. Male sterile lines of sorghum are especially susceptible. In sorghum, the period of floral gaping occurs only for several hours in the morning, and then the lemma and palea close tightly over the ovary (Frederickson and Mantle, 1988). The stigmas, however, remain exposed and are an important avenue for infection. Conidia of *C. sorghi* germinate on stigmas within 16 h and grow down the styles and begin infecting the ovary within 4 days (Frederickson and Mantle, 1988). Drops of honeydew exude from infected ovaries within 8–10 days after inoculation (Frederickson and Mantle, 1988), and contain huge numbers of conidia from sphacelial fructification at the base of the ovary. Sclerotia develop over a 4–6-week period (Frederickson and Mantle, 1988). The sclerotia are elongated, curved or straight, initially cream to buff, darkening to gray to light brown (Frederickson et al., 1989, 1991). Sclerotia incubated on moist sand produce stromata within 5 weeks (Frederickson et al., 1991). The perfect state was first described by Kulkarni et al. (1976) but later described in greater detail by Frederickson et al. (1991). Stipes
are bronze or deep terracotta, darker near the capitulum, white near the sclerotium. Capitula are buff, 0.7 mm in diameter, with a white collar at the base. Perithecia are 130–250 × 60–125 μm with asci of 56–114 × 2.4–3.2 μm. Ascospores are 40–97 × 0.4–0.8 μm. Macroconidia are oblong to oval, slightly constricted in the center, 8–19 × 4–6 μm. Microconidia are spherical, 2.5 μm in diameter. Ergoline alkaloids have not been detected (Frederickson et al., 1991).

Sangit Rao and Moghe (1995) reported finding a strain from *Dicanthium carsoma* L., which produced triangular conidia, a character that was retained when the strain was used to infect *Sorghum*. However, Langdon (1950) believed that the triangular conidia on *Dicanthium* species in India were due to infection from *Claviceps pussila*, and confirmed its presence in India in 1952 (Langdon, 1952).

### 2.36. *C. sorgicola* Tsukib., Shiman. & Uematsu

In 1999 a third species of *Claviceps* from *Sorghum* was reported, *Claviceps sorgicola* (Tsukiboshi et al., 1999). It is known only from Japan, and hosts include *Sorghum bicolor* (L.) Moench and *Sorghum sudanense* (Piper) Stapf. It differs from *C. sorghi* and *C. africana* in the production of tricyclic alkaloids similar to paliclavine, smaller conidia, absence of microconidia and secondary conidia, purplish black sclerotia, and larger ascospores (Tsukiboshi et al., 1999). Sclerotia are 2.5–20 × 1.9–3.5 mm, conical to cylindrical, straight or curved, purple-black to black. Stipes are brown to bronze. Capitula are globose to subglobose, 0.5–1.6 mm in diameter, dark brown, and distinctly papillate. Perithecia are ovate to pyriform, 215–300 × 105–140 μm. Asci are 122–215 × 2.5–3.8 μm with ascospores of 92–205 × 0.5–1 μm. Conidia are ellipsoid to oval, 5–11.3 × 2.5–3.8 μm (Tsukiboshi et al., 1999).

*C. africana* and *C. sorghi* infect primarily male sterile lines, whereas *C. sorgicola* occurs also in commercial fertile sorghum and Sudan grass (Tsukiboshi et al., 1999). Thus, *C. sorgicola* has the potential for spreading across a much broader range of *Sorghum* cultivars and areas of production.

### 2.37. *C. sulcata* Langdon

*C. sulcata* occurs on *Brachiaria* species in Southern Rhodesia, South Africa, and Uganda (Loveless and Herd 1964). It was originally described by Langdon (1954) but later described in greater detail by Loveless and Herd (1964). Sclerotia are gray-brown to almost black, up to 18 mm long × 2–2.5 mm wide, somewhat flattened, usually curved or twisted, with a conspicuous longitudinal groove on one side and a less conspicuous groove on the other side, and transversely or obliquely striate. Germination occurs after a resting period of about 6 months, and low temperature is not required to induce germination. Stipes are bright orange, becoming paler as the capitulum matures; a tuft of white hyphae persists at the base. Capitula are 1–2.5 mm in diameter. Langdon (1954a) described...
the capitula as straw-colored, while Loveless and Herd (1964) noted that they were bright orange. Perithecia are elliptic, 240–340 × 80–170 μm. Asci are 120–210 μm with ascospores of 110–195 × 0.5–1.0 μm. Conidia are allantoid, some cylindric or elliptic, 7.5–19 × 3–5 μm.

In 1995 *C. sulcata* was reported from *Brachiaria* species from Brazil. *Brachiaria* was introduced to Brazil from Africa as forage for livestock, and *C. sulcata* is believed to have been introduced with *Brachiaria* (Fernandes et al., 1995).

### 2.38. *C. tripsaci* Stevens & Hall

*C. tripsaci* was described from *Tripsacum dactyloides* (L.) L. from the southeastern United States. Sclerotia are initially white, becoming brown or black at the distal end, nearly conical, 4–5 mm in diameter at the base. Stipes are whitish purple, bearing gray to grayish white capitula. Perithecia are elliptical, 390 × 153–187 μm. Asci are 145–175 × 2–3 μm, with ascospores of 130 μm. Conidia are hyaline, fusoid to lunulate, 17.4–37.7 × 2.0–8.7 μm (Fig. 1v). Except for the size of the sclerotium, *C. tripsaci* is morphologically similar to *C. gigantea*.

### 2.39. *C. uleana* P. Henn.

*C. uleana* is based on a collection fruiting on the ground from seeds of a *Panicum* species in St. Catharina, Brazil (Henning, 1899). The sclerotia are dark brown, 1–1.5 × 0.5–0.8 mm. The stipes and capitula are gray to flesh-colored. Perithecia are 120–135 × 65–75 μm, with ascospores 60–70 μm long.

### 2.40. *C. viridis* Padwick & Azmatullah

*C. viridis* is known from *Oplismenus compositus* (L.) Beauv. from India (Watts-Padwick and Azmatullah, 1943) and *O. undulatifolius* Roem. and Schult. from Japan (Tanda, 1992b). Sclerotia are 1.9–8.1 × 0.8–1.9 mm, cylindric, obclavate or fusiform, blackish purple with a sulfurous green tint on the lower half (Tanda, 1992b). Stipes are yellow or light olivaceous. Capitula are globose or depressed globose, yellowish green to yellowish brown, 0.5–1.7 × 0.7–2.4 μm. Perithecia are immersed entirely in the capitulum, ovoid, oblong or obpyriform, 197–377 × 111–180 μm. Asci are 137–252 × 2.1–3.5 μm with ascospores of 130–182 × 1 μm. Macroconidia are *bacilliform* or cylindrical, 8.0–17.3 × 2.6–4.1 μm. Microconidia are ovate, 3.5–6.7 × 2.3–3.3 μm (Tand, 1992b). *C. viridis* is unique in that the sclerotia, conidia, stromata, and cultured mycelium have a greenish tint.
2.41. *C. yanagawensis* Togashi

*C. yanagawensis* was first reported from *Zoysia japonica* Steud. in Japan (Togashi, 1936). Sclerotia are depressed-cylindrical with accumulate to rounded ends, curved, 1.5–15 mm long × 0.55–1.3 mm wide, pale yellow green when immature, black, red-violet, or dark violet when mature. Stipes are dark brown, 0.7–6.2 mm. Capitula are subglobose, 0.3–1.0 × 0.4–1.5 μm, pale red-brown when immature, dark purple to brown-black when mature, with ostioles slightly raised. Perithecia are elongate-obovate, 180–320 × 70–190 μm. Asci are 85–165 × 4–8 μm with ascospores of 75–135 × 1–2.25 μm (Togashi, 1936). Conidia are ovoid or spindle-shaped, 11 × 5.4 μm (Langdon, 1952a).

2.42. *C. zizaniae* (Fyles) Pantidou

*Claviceps* on wild rice (Zizania species) was believed to be *C. purpurea* var. *purpurea* until infection studies by Fyles (1915) and later by Steinmetz and Wright (1943) revealed that it was a separate species. Sclerotia are cylindric, pointed to curved at one end, tan to brown, 5–16 mm × 3–6 mm (Fig. 2e) (Pantidou, 1959). Sclerotia harvested in September and placed outside under Canadian conditions in October germinated from December through May, with spores emerging from late December until mid-June (Fyles, 1915). Stipes are tawny to light vinaceous lilac, and glabrous (Pantidou, 1959). Capitula are spherical, 1–3 mm in diameter, tawny to russet in color, with brown perithelial ostioles (Figs. 3e and 3f). Perithecia are 200–330 × 90–200 μm. Asci are 110–226 × 3.5–4.5 μm with ascospores of 115–190 × 0.5–1 μm. Conidia are variable in size and shape, oblong-elliptic, reniform, some near-pyriform, 6.5–30 × 3–6 μm (Pantidou, 1959).

The only known host, wild rice, prefers shallow water, and its range includes southern Canada and the northern tier of the United States from Main to Nebraska (Hitchcock, 1971). The sclerotia float in water, a useful adaptation for an ergot in an aquatic environment. In Maine, infections of up to 1% were observed, with greatest severity in coves and drift areas (Steinmetz, 1940). Fyles (1915) found that sclerotia buried in mud or sand germinated well, but those left floating on water were overrun by molds and most perished, indicating that sclerotia washed up and buried on the shore or sandbars are best suited for survival and germination.

3. **UNCERTAIN OR INVALID SPECIES**

3.1. *C. flavella* (B. & C.) Petch

In 1869, Berkeley and Curtis described *Cordyceps flavella* from Cuba, as a species apparently growing on an uncharacterized sclerotium among leaves and
wood, producing yellow stipitate stromata with globose capitula, prominent perithecia, and slender asci. In 1895 Massee redescribed *C. flavella*, adding that the stipes were glabrous, capitula were 2 mm in diameter, ascospores were multiseptate, and it was growing from a portion of a caterpillar. His illustration clearly shows the stromata developing from the body of a caterpillar. In 1920, Lloyd noted the material at Kew was mostly gone, but was able to view a specimen at Paris that Berkeley had send to Montagne (Lloyd, 1920). A pale yellow stem and capitulum were described as growing from a geometrid (Lloyd, 1920). In 1933 Petch also noted the material at Kew was scant, but believed a collection by Duss in 1903 from Guadeloupe was equivalent to the type material (Petch, 1933). However, whereas the collection described by Berkeley and Curtis was growing from an insect, the collection observed by Petch (1933) was growing on and surrounding a grass spikelet. To complicate matters further, Petch (1933) not only transferred *Cordyceps flavella* to *Claviceps flavella* but stated that it was conspecific with *Claviceps balansoides*. The description provided by Petch (1933) does not match that of Berkeley and Curtis (1869) for *Cordyceps flavella* or of Möller (1901) for *C. balansoides* (see additional discussion under *C. balansoides*). Therefore, the status of *C. flavella* (B. & C.) Petch must be held with uncertainty.

### 3.2. *Claviceps junici* J. F. Adams

Adams (1907) found the sphacelial stage of presumably a *Claviceps* infecting the ovaries of *Juncus glaucus* Sibth. The conidia are oblong to elliptical, 7–10.3 × 2.8–3.5 µm. Although various authors have used the name *C. junici*, it is invalid since description was based only on the occurrence of the sphacelial stage.

### 3.3. *C. rubra* Whetzel and Reddick


### 3.4. *C. synthetismae* Swade

Swade (1944) proposed the name *C. synthetismae*, although this is invalid since the description was based only on the anamorphic state. Tanda (1992a) mentions that sclerotia of *C. amaniensis* are similar to those described by Sawade (1944) in Formosa, and it is possible that they could be conspecific.

### 3.5. *C. typhoides* Sulaiman, Lukade and Dawkhar

Listed in Lenné (1990) as occurring on *Pennisetum typhoides* (Burm. F.) Stapf and CE Hubbard from India, based on records at IMI.
4. SPECIES NOT RECOGNIZED AS CLAVICEPS

4.1. *Balansia claviceps* Speg. (= *C. philippi* Rehm)

Rehm (1889) described a collection from Mergui, Chile, although the host was not identified. Sclerotia are 2–2.5 cm long, black, subcylindrical, developing within infected caryopses and on the inside of leaf sheaths. Stromata are stipitate, scabrous, and capititate. Capitula are 0.3–1 mm in diameter. Asci are 120–150 × 6 μm with ascospores of 120 × 1 μm. Rehm (1898) later identified *C. philippi* as a synonym of *Balansia claviceps*. The synonymy was confirmed by Diehl (1950).

4.2. *Balansia pallida* Wint. (= *C. patouillardiana* (Pat.) P. Henn.; = *C. pallida* Pat.; = *C. pallida* (Wint.) P. Henn.)

*Balansia pallida* was described from *Luziola peruviana* Juss. ex J. F. Gmel. from St. Catharina, Brazil. As described by Winter (1887), stromata are gregarious, sclerotia-like, bulbous or subglobose, 0.5–0.2 mm wide, sessile or stipitate, and pale yellow. Stipes are yellow-white, bearing capitula 0.3–0.2 cm wide, with prominent perithecia. Perithecia are elongate-ovate, 290–320 × 130–160 μm. Asci are 175–220 × 3.5–4 μm with ascospores of similar length and 0.8–0.9 μm wide. Stylospores are 44–62 × 2 μm. Henning (1899) believed that *B. pallida* was more closely aligned with *Claviceps*, a view not shared by Rehm (1900). Diehl (1950) referred it back to *Balansia*, based on production of ephelidial fructifications.

4.3. *Neobarya aurantiaca* (Plowr. and A. S. Wilson) Rauschert (= *Baryella aurantiaca* (Plowr. and A. S. Wilson) Rauschert; = *Barya aurantiaca* Plowright and Wilson; = *Claviceps wisonii* Cooke)

In 1884, Plowright and Wilson described the development of *Barya aurantiaca*, parasitic on sclerotia of *Claviceps* from *Glyceria fluitans* (L.) R. Br. Stromata are vertical, clavate or subclavate, 10–20 mm high × 1–3 m wide. Immature stromata are floccose, white with conidiferous hyphae. Yellow perithecia with orange ostioles are produced in the upper two-thirds. Conidia are elliptical-lanceolate, 10–12 × 2–3 μm, borne in chains on the ends of branching conidiferous hyphae. Perithecia are 250–300 × 150 μm with asci 250 × 30 μm. Fruiting bodies of *C. purpurea* var. *purpurea* and *Neobarya* may arise from the same sclerotiun, but typically, infected sclerotia bear only the fruiting bodies of *Neobarya*. In infection studies, the conidia of *Neobarya* would not establish infections of *G. fluitans* (Plowright and Wilson, 1884).

Cooke (1884), apparently unaware of the publication of Plowright and Wilson (1884), described what he believed to be a new species of *Claviceps*, *C. wisonii*, from *G. fluitans*. His description of the species (Cooke, 1884) is in
complete agreement with *N. aurantiaca*, and there is no doubt that Cooke mistakenly described *Neobarya* as *Claviceps*.

### 4.4. *Sclerotinia duriaeana* (Tul.) Rehm (= *Claviceps caricina* D. Griffiths)

Griffiths (1902) listed this species as *Claviceps caricina*, primarily with the objective of calling attention to it for further study. The description is limited to the sclerotia, which were produced within the stems of *Carex nebraskensis* Dewey. Groh (1911) identified it as *Sclerotium sulcalum*. Type material was later examined by Whetzel (1929), who recognized *S. sulcatum* as synonymous with *Sclerotinia duriaeana*.

### 4.5. *Ustilaginoidea virens* (Cooke) Takah. (= *Claviceps oryzae-sativa* Hashioka; = *Claviceps virens* Sakurai; = *Ustilago virens* Cooke; = *Tilletia oryzae* Patouillard; = *Sphacelotheca virens* (Cooke) Omori; = *Ustilaginoidea oryzae* Brefeld)

Infection of rice by *U. oryzae* is commonly referred to as false smut. The disease has been reported from almost all rice-growing regions in the world (Hashioka, 1971). The fungus was first described as an *Ustilago* (Cooke, 1878), since conidiation resembles that found in *Ustilago*. It was believed to be a *Tilletia* by Patouillard (1887) and a *Sphacelotheca* by Omori (1896), who thought it to be a true smut. Infected ovaries are transformed into large velvety masses (pseudo-morphs) about twice the size of the normal grain (Hashioka, 1971). A technical description of *U. virens* was provided by Hashioka (1971). In cross section the pseudo-morphs consist of a dense core of mycelium, surrounded by a whitish yellow layer, which is surrounded by an orange-yellow layer, surrounded again by an olive-black outermost layer. Conidia are subglobose, 4–6 × 3–5 μm, yellowish at first, turning dark olive-brown with maturity. The epispore is 0.3 μm thick with a prominent dark olive laciniate ornamentation. Secondary conidia are subglobose, globose to oblong, 4–8 × 2–5 μm. One to two sclerotia develop within the center of each pseudo-morph. Sclerotia are 5–13 × 2–5 mm, black, variably shaped. Sclerotia germinate after 4–5 weeks at 24–30°C. Capitula are 1–3 mm in diameter, dark greenish yellows to olive. Perithecia are ovate to pyriform. Ascospores are 50–80 × 0.5–1 μm (Hashioka, 1971).

Brefeld (1895) believed that an ascigerous state would arise from the pseudo-sclerotia and erected the genus *Ustilaginoidea* to accommodate it, placing it as *Ustilaginoidea oryzae*. Sakurai was the first to observe the ascigerous state but incorrectly applied the anamorphic epithet virens, as *Claviceps virens* (Ou, 1985). Hashioka (1971), believing the fungus to be a true *Claviceps*, defined it as *Claviceps*.
oryzae sativa. However, the formation of ustilaginoid conidia places the fungus outside Claviceps. The fungus is currently referred to in the anamorph form as Ustilaginoidea virens (Cooke) Takah. (Takahashi, 1896).

5. MORPHOLOGICAL DIVERSITY

Conidia of Claviceps species are typically hyaline, single-celled, thin-walled, and may contain guttules. Shape of conidia varies widely, including spherical, ovate, oblong, fusiform, cuneate, or triangular (Fig. 1). In some cases, with ovate to cylindric conidia, a slight constriction may occur in the center of the spore. Depending on the species of Claviceps, up to three different types of conidia can be produced: microconidia, macroconidia, and secondary spores. Size of microconidia is proportional to the macroconidia, often a quarter to a third the size of the macroconidia. Secondary spores are produced from the germination of macroconidia. In species such as C. fusiformis a wide range of conidial sizes occurs through the production of macroconidia that can germinate to produce secondary conidia that in turn can undergo budding. The process of conidiation has been described in detail for species such as C. africana, C. fusiformis, C. paspali, and C. purpurea, but for many of the species it is not clear if more than one conidial type occurs. Some species (e.g., C. zizaniae, C. tripsaci, C. viridis) have a very wide range of conidial sizes. In C. viridis, length of conidia from honeydew can range from 4.2 to 18.9 μm (Watts-Padwick and Avmatulla, 1943). However, Tanda (1992) differentiated macro- and microconidia produced in culture. Thus the wide range in size among some species of Claviceps may be due to the presence of macro- and microconidia, which would be difficult to distinguish if their range of sizes overlapped. Although conidial shape and size may vary widely among species of Claviceps, conidial size and shape are relatively stable within species.

Sclerotia among species of Claviceps can vary in size, coloration, and surface morphology. Generally, the size of the sclerotium is proportional to the size of the host seed, with larger sclerotia occurring in larger-seeded plants, often 1–4 times the size of the host seed. Basic shapes range from spherical to oblong to elongated with rounded or tapering ends, or more variable as in the mycelial-sclerotia type such as C. balansoides or C. orthocladae. Surface texture can vary from a matte to coarse, rugose, or fissured. Color varies from white (in the case of albino variants) to yellow, gray, brown, green-tinted, dark purple, purple-black, to black. In some cases a blackish appearance can occur from the superficial growth of secondary fungi.

The sclerotium is initiated following the development of a sphaecelium on the outer surface of the ovary. As the structure enlarges, the internal mycelium converts to parenchymatous tissue and the sphaecelium is incorporated into the rind. As sclerotia mature, the outer layer (the rind) may remain smooth or become roughened or wrinkled. In elongated sclerotia, such as in C. purpurea var. purpurea,
longitudinal furrows may appear along the surface, and the apex may appear rugose. At the apex of sclerotia it is not uncommon to find remains of some floral parts, e.g., stigmas, which remain as the fungus proliferates and develop below the apex. In spherical sclerotia, e.g., *C. paspali*, the surface may appear cracked and fissured. In the case of mycelial-sclerotia types the sclerotia are variable in size and shape (Figs. 2a–2e), may include parts of the floret, and may be black or yellow. Sclerotial morphology is a stable attribute within species of *Claviceps*.

In sclerotial germination, one to many stipes can develop. In general, larger sclerotia can support a greater number of stromata. Langdon (1952a) was especially interested in the initial stages of sclerotial germination and placed some emphasis on whether a tuft of hyphae developed at the base of the stipe, and its presence or absence is believed to be a stable attribute within a species. As stipes develop, the outer surface may be smooth or glabrous, depending on species. Stipe color varies greatly, including lucid, white, yellow, tan, light purple to dark purple, or near-black. Stipe length can vary greatly depending on light conditions, with longer stipes produced in the dark. In some species, e.g., *C. annulata*, a ring of hyphae (annulus) may occur on the stipe, just below the capitulum. The connection to the stipe to the capitulum can occur at the base or up to halfway into the capitulum.

Capitula in *Claviceps* are typically globose to subglobose, often in the size range 0.5–1.5. Color spans a broad spectrum, including white, yellow, gray, tan, brown, dark purple to purplish black, or near-black. Color of the stoma is considered one of the most important characters for species differentiation (Langdon, 1954). However, whereas Langdon (1954) described the capitula of *C. sulcata* as straw-colored, Loveless and Herd (1964) noted that they were bright orange. Many of the species are based on relatively small collections and the variability in color within a given species may not be fully realized.

Langdon (1942) believed that size of perithecia was a stable attribute for species separation. However, variation in size of perithecia can occur depending on how measurements are taken. Herd and Loveless (1965) noted the smaller dimension of the perithecia reported by Hansford (1940), but believe they arrived at a higher value by taking only median sections through fully mature capitula. Of greater concern is the case of *C. rolfsii*. If one accepts *C. rolfsii* as synonymous with *C. paspali*, where there is a very large difference in size of perithecia between the two, then one can no longer place value on the size of perithecia as a useful character for species separation. If perithecia can vary in size, then one would expect the asci also to vary, and this is indeed true. The extent of the protuberance of perithecia from the capitulum has been considered an important element in the separation of species. In some species the perithecia appear only partially exposed, while in others, such as *C. ranunculoides*, they are almost completely exposed. However, extent of emergence from the stroma can vary within species and with maturity. In *C. zizaniae*, for example, a wide range
in extent of protuberance of perithecia from the stromatal surface can occur (Figs. 1e and 1f).

Möller (1901) recognized unusual patterns of ascospore germination, such as repeated conidia formation, and disarticulation of ascospores. Observations on ascospore germination have not been included in the technical description of most *Claviceps* species, although such observation may have some value in characterizing and differentiating some species.

6. DIVERSITY IN HOST RANGE

Host range among *Claviceps* species was summarized by Kawatani (1953), Grasso (1957), and Brady (1962). Host range is among the most important characters in the recognition and separation of species of *Claviceps*. Many of the *Claviceps* species are believed to be restricted to a single genus or closely related genera, although the full extent of host range for many of these species has not been fully investigated. For some species, e.g., the sorghum ergots (*C. africana, C. sorghi*, and *C. sorgicola*), host range can overlap. *C. purpurea* var. *purpurea* has the largest host range, believed to exceed 400 species (Bove, 1970), but restricted largely to the subfamily Pooideae. Unfortunately, most host reports of *C. purpurea* were based on the assumption that *C. purpurea* was the causal agent and only very rarely were sclerotia germinated to verify the fungal identity. In particular, reports of *C. purpurea* var. *purpurea* on panicoid hosts are likely to be in error.

*C. purpurea* var. *purpurea* is genetically diverse (Jungehülsing, 1995; Tudzynski, 1999), and host-inoculation studies have shown conflicting results among isolates *C. purpurea* var. *purpurea*, due not only to variation among strains but to techniques used for inoculation (Campbell, 1957). Diversity among strains of *C. purpurea* var. *purpurea* can be enhanced through the widespread distribution of ergot with seed, including the cereal grains, and grasses for forage, turf, reclamation, or ornamental uses. It is of particular interest to note the occurrence of mitochondrial plasmids from wild strains of *C. purpurea* var. *purpurea* (Tudzynski and Esser, 1986). Oeser and Tudzynski (1989) suggested the possibility that such plasmids may be associated with host specificity.

In evaluating host range, especially for species identification, one must consider variability among strains, methodology, and the possible confounding effect of overlapping host range. Advances in our understanding of host diversity in *Claviceps* will require an increased understanding of the molecular genetics of host specificity.

7. ECOLOGICAL DIVERSITY AND ADAPTATION

*Claviceps* species have evolved mechanisms for survival and timing of ascospore production in diverse habitats. In temperate climates, species such as *C. purpurea,*
C. paspali, C. grohi, and C. zizaniae require low-temperature exposure for germination. This parallels the vernalization requirement of many of the cool-season grasses and serves as a mechanism to prevent premature germination. In cool temperate climates, e.g., the northern United States, sclerotia of C. purpurea are resistant to decay, but in warmer temperate climates, e.g., the southern United States, the sclerotia are subject to fungal and bacterial colonization and destruction (Cunfer and Seckinger, 1977). C. paspali, however, which occurs on Paspalum species in the southern United States, is more resistant to microbial colonization and decay (Cunfer and Seckinger, 1977).

In the semiarid tropics, Claviceps species are adapted to warm temperatures. The sclerotia of C. fusiformis, for example, require a storage temperature of 20–37°C before germination (Prakash et al., 1987). Prakash et al. (1987) concluded that high temperatures combined with periods wetting and drying simulate the premonsoon conditions of semiarid tropics and would support good germination of sclerotia. Unlike C. purpurea, which requires cold temperatures to stimulate germination, Prakash (1987) found that chilling of sclerotia of C. fusiformis actually reduced the percentage of germination.

The adaptation of Claviceps to aquatic grasses varies, depending on temperature. In the case of C. zizaniae, which attacks the freshwater grass Zizania, a cold conditioning is required, as would be expected for a northern-climatic ergot. However, C. purpurea var. spartinae, which colonizes grasses along warm and temperate coastal waters with temperatures, especially along the Gulf coast, where winter conditions are warm, does not require a cold conditioning period. For both C. zizaniae and C. purpurea var. spartinae, the sclerotia wash onto sandbars or shorelines, they must be partially buried to facilitate germination, an adaptation which also localizes the sclerotia in the vicinity of the host.

In arid habitats, fungi such as C. cinerea, C. citrina, and C. maximensis germinate quickly in response to moist soil conditions. Whereas cold-climate fungi such as C. grohi may take 10 weeks or more to germinate, the fungi adapted to arid habitats germinate and produce mature fruiting bodies within a month, coinciding with the rapid germination and reproduction of host grasses. A cold conditioning is not required.

Local populations and species of Claviceps that have evolved within a region can be introduced into other areas through the international seed trade. The recent global dissemination of C. africana and the transfer of C. sulcata from Africa to Brazil during the introduction of Brachiaria species (Fernandes et al., 1995) illustrates the potential for the introduction of Claviceps species into new areas. With an introduction of a new species there is the potential not only for an expansion of host range but for the displacement of one species by another. Such a scenario is believed to have occurred in the displacement of C. sorghi by C. africana in India (Pazoutová et al., 2000a). One can also not completely rule
out the possibility of coinfection or hybridization among native and introduced strains or species.

The role of insects is very important in the life cycle of genera closely related to *Claviceps*. In *Epichloë* the stromata and conidia develop on the leaf culms. Although not sugary as with the honeydew of *Claviceps*, the hypothallus is nevertheless attractive to certain insect species that feed on it. In *Epichloë* conidia function as spermatia, where fertilization involves transfer of conidia of one mating type to a stroma of the opposite mating type for fertilization. Although it is not required for fertilization in *Claviceps*, transfer of conidia by insects may provide a means of genetic exchange among populations of *C. purpurea var. purpurea* (White et al., 2000). Swan and Mantle (1991) established that distinct strains of *C. purpurea var. purpurea* can coexist within a single sclerotium. Tudzynski (1999) found that most field isolates were heterokaryotic, which could occur through mixed infections and a generally high mutation rate. Thus insects may serve an important role in the intermixing of conidia as well as in disease spread.

Considerable diversity exists in the *Clavicipitaceae*, which provides attributes for differentiation among species of *Claviceps*. However, stability of attributes needs to be better understood. Single ascospore lines from the same sclerotium of *C. purpurea var. purpurea* show considerable variation in morphology in culture, in number of conidia produced in culture, and in both type and quantity of alkaloid produced (Esser and Tudzynski, 1978). Considerable variability has also been observed in the analysis RAPD (Jungelülsing et al., 1997). Although variability in *C. purpurea var. purpurea* is well recognized, the extent of variability among many of the other species of *Claviceps* is not well understood. Considerable research is needed, especially to understand molecular and genetic diversity in *Claviceps* and the application of this research to define the limits of genera and species within the *Clavicipitaceae*. Phylogenetics may be especially helpful in resolving speciation and relationships among species in the *Clavicipitaceae*. However, ITS sequence analyses has led to some unexpected results. Pazoutová (2001) reported that *C. purpurea var. purpurea* and *C. fusiformis* were 98.7% identical, despite the fact that the species differ widely in terms of morphology, physiology, and ecology. Tooley et al. (2001), however, reported 84.6% similarity among the two species based on the β-tubulin intron 3 region and 61.2% similarity for the EF-1α intron 4. Clearly, additional studies are needed in the application of molecular genetics to understanding the phylogenetic relationships in *Claviceps* and of other genera in the *Clavicipitaceae*.

Most of the species of *Claviceps* have been described based on a limited set of morphological or host susceptibility characteristics. Additional collections and more detailed study, including molecular and phylogenetic analyses, are needed to support or refute the current listing of known species of *Claviceps*. Copyright © 2003 Marcel Dekker
Concurrently, better understanding is needed of the morphological and genetic variability within and among populations, including collections from diverse geographic areas. Phylogenetic analyses are needed to help place the origin of *Claviceps* species, identify their geographic movements, and establish evolutionary relationships. Despite the volumes of research on *Claviceps*, considerable study is still needed to better understand speciation, and the morphological, geographic, physiological, genetic, and host diversity within *Claviceps* and within the Clavicipitaceae.

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Nigrocornus scleroticus, a Common Old World Balansioi Fungus

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1. INTRODUCTION

Prior to Diehl’s (1950) monograph of Balansia and the Balansiae, the literature on the balansioi genera was restricted predominantly to descriptions of new taxa. Diehl attempted to bring order to the taxonomy of the balansioi fungi. He recognized three tribes in the Family Clavicipitaceae Subfamily Clavicipitoideae, namely, Clavicipitaceae, Ustilaginoideae, and the Balansiae, that classification being based on fundamental differences in the anamorph of the members of each tribe. In the Balansiae (more correctly Balansieae), he included any fungus with the following characteristics: a basal stroma (hypothallus) covering or enclosing some part of the host, on which an anamorph referable to Ephelis Fr. and/or Neotyphodium Link. [= Acremonium Link. Sect. Albo-lanosa Morgan-Jones & Gams (Glenn et al., 1996)] developed, following by a teleomorph consisting of an effuse, capitate, or stipitate ascostromata with ostiolate perithecia.

Diehl (1950) assigned four genera, Atkinsonella Diehl, Balansia Speg., Balansiopsis Höhn., and Epichloe (Fr.) Tul., to the Balansieae. He also recognized two subgenera of Balansia, namely, Eubalansia Diehl and
Dothichloë (Atk.) Diehl. Diehl (1950) noted that the traditional criteria for distinguishing Balansia from related genera had been restricted to characteristics of the ascostroma and the structure from which it arose. Little attention had been paid to the role of “accessory fructifications,” despite the fact that a genetic relationship between Balansia and Ephelis had been previously established (Ellis and Everhart, 1886; Cooke and Masse, 1889). Although Diehl recognized the importance of the characteristics of the teleomorph in the taxonomy of Balansia and related genera, he considered that the location and morphology of the basal stroma and the anamorph were also useful taxonomic criteria.

Up until the late 1970s the balansioid genera were largely ignored, but the recognition of the toxic effects of some Balansia species stimulated research on the group. Luttrell and Bacon (1977) and Rykard et al. (1982) provided conclusive evidence that Myriogenospora Diehl should be placed in the Balansieae, and Dussiella Pat. (=Echinodothis Atk.) was added to the tribe by White (1993). Diehl (1950) had considered that the lack of anamorphs in fungi assigned to Balansiopsis Höhnel was an important taxonomic criterion, but Ephelis anamorphs have now been described for all of them (White, 1997).

White (1997) has questioned the separation of the Clavicipitaceae into tribes, and has recognized eight genera and subgenera of graminicolous clavicipitatean fungi, Balansia subgenus Eubalansia, Balansia subgenus Dothichloë, Myriogenospora, Atkinsonella, Ustilaginoidea Bref., Claviceps Tul., Echinodothis, and Epichloë (Fr.) Tul. White (1997) considered that the relationship of the hyphae with the host tissues (that is, epiphytic or endophytic), in addition to the features used by Diehl (1950), was also an important taxonomic feature. Since then, Parepichloë White and Reddy (1998) has been erected to accommodate epiphytic fungi with brown to black ascostromata on warm-season grasses. No anamorph was described for any of the seven species assigned to the new genus. Neoclaviceps White, Bills, Alderman and Spatafora (Sullivan et al., 2001) was erected to accommodate a fungus that produced a stipitate ascotroma from a basal stroma in the ovary of its hosts, and although the anamorph was described as ephelidial some of its features differ significantly from those of Ephelis.

Kuldau et al. (1997) conducted phylogenetic studies of clavicipitatean fungi and identified five clades, one including species of Atkinsonella, Balansia, and Myriogenospora (the “Ephelis clade”), and others whose asexual conidia are enteroblastic (Sphacelia Lev., Sphacelia-like, or Neotyphodium), such as species of Claviceps, Cordyceps (Fr.) Link and Epichloë.

In this chapter, only clavicipitatean genera whose anamorphs belong in Ephelis (the balansioid genera) are discussed further. The characteristics of the clavicipitatean genera are discussed in Chapter 7 of this volume.
2. CHARACTERISTICS OF BALANSIOID GENERA

2.1. Hyphae

Some balansioid fungi, e.g., _Balansia claviceps_ Speg., _Balansia epichloë_ (Weese) Diehl. [= _Balansia kunzei_ Morgan-Jones and Phelps (1995)], _Balansia henningsiana_ (Möller) Diehl, _Balansia nigricans_ (Speg.) White, Drake and Martin, _B. obtecta_ Diehl, and _Balansia strangulans_ (Mont.) Diehl, invade host tissues, the hyphae being endophytic (Rykard et al., 1985; Leuchtmann and Clay, 1988; Clay and Frentz, 1993; White et al., 1996; Reddy et al., 1998). Epiphytic relationships between hyphae and host tissues have been reported for others, such as _Atkinsonella hypoxylon_ (Pk.) Diehl (Leuchtmann and Clay, 1988), _A. texensis_ (Diehl) Leuchtmann and Clay (1989), _Balansia andropogonis_ Syd. & Butler (Reddy et al., 1998), _Balansia cyperi_ Edg. (Clay, 1986a,b), _B. pallida_ (Wint.) Diehl (Clay and Frentz, 1993), _Balansia pilulaeformis_ (Berk. & Curt.) Diehl, and _Myriogenospora atramentosa_ (Berk. & Curt.) Diehl (Rykard et al. 1985). A study by Smith et al. (1985) proved that there was reciprocal translocation of carbohydrates between hyphae of _M. atramentosa_ and the host’s cells. White et al. (1991) found that _A. hypoxylon_ had a greater ability to utilize oil and paraffin compared to endophytic balansioid fungi, and surmized that it could utilize the cuticle as an energy source. Leuctmann and Clay (1988) hypothesized that the close association between hyphae of _A. hypoxylon_ and _B. cyperi_ and plant cells undergoing rapid cell division could enhance the ability of the fungi to utilize nutrients from the host and also influence plant growth and development through the production of phytohormones by the fungi.

2.2. Anamorph

The conidiomata of _Ephelis_ develop on a basal hyphal stoma in association with various host organs. The basal stromata of some species, for example, _B. claviceps_, _B. obtecta_, _B. andropogonis_, and _B. oryzae-sativa_ Hashioka, consist of an underdeveloped inflorescence bound by hyphae into a spindle-shaped structure. The basal stroma of the _Ephelis_ anamorph of _Balansia pallida_ develop within the florets of host inflorescences, while those of _Myriogenospora_ consist of leaves tightly rolled by hyphae. By contrast, the basal stromata of _Balansia_ species assigned by Diehl (1950) and White (1997) to the subgenus _Dothichloë_ develop as an effuse layer on culms, such as _B. strangulans_, and _Balansia aristidae_ (Atk.) Diehl, or on the surfaces of leaves, such as, _B. epichloë_, _B. henningsiana_, and _Balansia linearis_ (Rehm) Diehl (White, 1997).

The conidiomata of balansioid fungi vary markedly in morphology. At one extreme are the discrete, cupulate-patellate structures referred to as being apothecia by Rykard et al. (1982, 1984), sporodochia by Morgan-Jones and White (1989), and acervuli or pycnidia by Diehl (1950). Such structures are
characteristic of the following fungi: *A. hypoxylon* (Rykard et al., 1984), *B. claviceps*, *B. obtecta*, and *B. cyperi* (Diehl, 1950), *B. oryzae-sativae* (Tai and Siang, 1948), *B. pilulaeformis* (Rykard et al. 1984), and *M. atramentosa* (Rykard et al., 1982). Critical studies by Rykard et al. (1984) and Morgan-Jones and White (1989) have shown that this type of conidioma is erumpent from beneath the surface of the stroma. At the other extreme are the conidiomata of *B. epichloë* and *Balansia gaduae* (Rehm.) J. F. White, which consist of an effuse layer of conidiophores covering the basal stroma (White et al., 1997). Between these extremes several other forms have been described, such as the elongate or hysteriform conidiomata of *B. aristidae*, *B. henningsiana*, and *B. strangulans* (White et al., 1996, 1997).

There is evidence of another type of ephelidial fructification. Hashioka (1971) reported that the pycnidial conidiomata of *B. oryzae-sativae* developed on a stroma surrounding the host’s inflorescence. On some tillers which ultimately bore such a stroma, a lustrous, grayish-white coating often covered the upper leaf blades. Other workers (Venkatakrishnaiya, 1946; Govindu and Thirumalachar, 1961; Mohanty, 1964) noted a similar occurrence on tillers infected by *Ephelis oryzae* Syd. Although Ramakrishnan et al. (1953), Govindu and Thirumalachar (1963), and Janardhanan et al. (1991) reported that ephelial fructifications occurred on the upper leaves of tillers bearing ascostromata of *B. sclerotica* (sic), they did not prove that the two fructifications were related. Studies in Queensland, Australia, and Japan have demonstrated conclusively that the conidia of some *Ephelis* species develop in acervuloid conidiomata on stromata which envelop the host’s inflorescence, and on effuse hyphal layers on the upper leaves of the same tillers (Ryley, unpublished data; Christensen et al., 2000).

It is widely accepted that the conidia of *Ephelis* species are acicular, hyaline, guttulate, straight or slightly curved, and aseptate, and that they develop on simple or branched conidiophores (Diehl, 1950; Rykard et al., 1982, 1984; Leuchtmann and Clay, 1989; Morgan-Jones and White, 1992; Phelps et al., 1993; Phelps and Morgan-Jones, 1993; White, 1997). Conidium ontogeny has been described for a few *Ephelis* species. Rykard et al. (1982, 1984) and Morgan-Jones and White (1989, 1992) have studied the conidium ontogeny of *A. hypoxylon*, *A. texensis*, *B. epichloë*, *B. henningsiana*, *B. pilulaeformis*, *B. strangulans*, and *M. atramentosa*. They found that the first conidium is acrogenous, and that before the conidium reaches its full size a new growing point in the conidiophore wall located below and to one side of the first conidium produces a lateral proliferation, at the tip of which a second holoblastic conidium develops. The conidiophore elongates for a short distance, and the pattern of conidium production is repeated. Although this process results in a geniculate extension of the conidiogenous cell, the sympodium is so short that the conidia appear to be whorled. Conidium production for the anamorphs of *B. aristidae* and *B. gaduae*...
has been described as holoblastic at geniculate apices (Phelps et al., 1993; White et al., 1997).

In their description of the anamorph of Neoclaviceps monostipa White, Bills, Alderman & Spathafora, Sullivan et al. (2001) reported that new conidia budded holoblastically from sypodial proliferations at the apices of the conidiophores. The conidia were 36–72 μm long and 1–3-septate. They assigned the anamorph to Ephelis, despite the fact that all other descriptions of Ephelis anamorphs, apart from that of Balansia linearis (Rehm) Diehl (Diehl, 1950), refer to the conidia as being aseptate (Diehl, 1950; Rykard et al., 1984; Phelps and Morgan-Jones, 1993; Phelps et al., 1993), and that conidium length is <40 μm (Morgan-Jones and White, 1992; Phelps et al., 1993; Phelps and Morgan-Jones, 1993; White et al., 1996, 1997).

2.3. Teleomorph

For all balansoid fungi with an Ephelis anamorph (except those in Parepichloë), the ascostromata develop on or in the stoma which bore the Ephelis fructifications toward the end of and/or after conidium production (Diehl, 1950; Rykard et al., 1982, 1984; Morgan-Jones et al., 1993). However, ascostomata of Balansia or related genera do not always develop on Ephelis conidiostromata (Govindu and Thirumalachar, 1961; Venkatakrishnaiya, 1946; Morgan-Jones and Phelps, 1995; personal observations).

Although the mature ascostromata of all species in Atkinsonella, Balansia, and Myriogenospora are black, they vary considerably in morphology. The ascostromata range from the narrow, linear stromata in curled leaves of Myriogenospora to the pulvinate, stipitate types of B. andropogonis, B. claviceps (Reddy et al., 1998), and B. obtecta Diehl (Diehl, 1950). Others, such as, A. texensis, B. cypéri, B. obecta, and B. pallida, are hemispherical-pulvinate and sessile-subsessile, or flat-pulvinate, such as A. hypoxylon, B. aristidae and B. strangulans (Diehl, 1950; Leuchtmann and Clay, 1989; Morgan-Jones et al., 1993). In B. epichloë and B. henningsiana, ascostoma cover the entire stroma, while in A. hypoxylon, B. aristidae, and B. pilulaeformis, the perithecia are restricted to certain areas and are separated by sterile areas (Rykard et al., 1984; Morgan-Jones et al., 1993).

The ontogeny and morphology of perithecia are essentially similar for all balansoid species studied, and have been described in detail for B. aristidae (Morgan-Jones et al., 1993), B. epichloë (Morgan-Jones and Phelps, 1995), and M. atramentosa (Luttrel and Bacon, 1977). At maturity, stromata have a layer of melanized cells at the outer surface, and one or more layers beneath, usually composed of pseudo-parechymatous cells. Perithecia are obpyriform-lageniform immersed or protruding, with walls consisting of elongate cells, persistent periphyses lining the neck, and asci and evanescent paraphyses at the base. Asci
are elongate, with a domed or flattened apex lined by a pore (such as in *A. hypoxylon*, *B. epichloe*, and *B. henningsiana* (Jones and Clay, 1987), or an apical thickening, such as in *M. atramentosa* (Luttrell and Bacon, 1977; Jones and Clay, 1987). Ascospores are filiform multispetate, and 7-septate (*A. hypoxylon*, *A. texensis* (Leuchtmann and Clay, 1989), *B. aristidae*, and *B. epichloe* (Morgan-Jones et al. 1993; Morgan-Jones and Phelps, 1995)). Within the ascus, ascospores may be straight (*A. hypoxylon* and *B. henningsiana*), or spiraled (*B. epichloe* and *M. atramentosa*) (Jones and Clay, 1987). The ascospores often break up into aseptate or 1-septate part-ascospores at maturity, sometimes while still within the ascus (White, 1997).

3. **NIGROCORNUS SCLEROTICUS GEN. NOV**

In contrast to the considerable research on the biology, development, and taxonomy of balansioid genera in the Americas, there have been few studies of similar fungi elsewhere. Literature on balansioid fungi outside the Americas is confined mainly to descriptions of new taxa, usually without a critical examination of related fungi.

During a study of systemic diseases of native grasses conducted in southern Queensland, Australia, since 1978, black, corniform, papillate ascostromata of a balansioid fungus were found surrounding the axillary buds on tillers of living tussocks of wild sorghum, *Sorghum leiocladum* (Hack.) Hubb. The fungus was identified at the Commonwealth Mycological Institute, Kew, England, as *Balansia sclerotica* (Pat.) Höhnel (Sivanesan, personal communication). Subsequently, the fungus has been found on five other perennial grasses (*Cymbopogon refractus* (R. Br.) Camus, *Entolasia stricta* (R. Br.) Hughes, *Paspalidium criniforme* S. T. Blake, *Paspalum scrobiculatum* L., and *Triodia triandra* Forsskal) in southern Queensland. From field and laboratory studies of the fungus and studies on herbarium material, it has become evident that the species *sclerotica* differs markedly from the type species of *Balansia* (*Balansia claviceps*) and from all other balansioid species. The genus *Nigrocornus* is proposed, with *Nigrocornus scleroticus* the only species.

3.1. **Historical**

*Epichloe sclerotica* Pat. was described on grasses from Fac-Bin, Tonkin, East Asia (China) by Patouillard (1890), who believed that the black, corniform ascostromata were on the inflorescences, surrounding the glumes of the spikelets. Hennings (1900) transferred the species to *Ophiadiothis*, and some years later Höhnel (1911) found that the ascostromata were associated with the axillary buds at the nodes of the culms, and not in the inflorescences. He described the ascostroma as consisting of the embryonic leaves of the buds enclosed and
covered by plectanchymatous hyaline, and bound on one side by the edges of the longest and outermost leaf (= protophyll) of the axillary bud. Höhnel (1911) considered that *Ophiodothis* was a synonym of *Balansia*, and transferred the species *sclerotica* to that genus.

Ramakrishnan et al. (1953) and Govindu and Thirumalachar (1963) noted that the dimensions of the ascostromata of *B. sclerotica* varied from host to host. In both reports, mention was made of an ephelidial state which occurred as a white deposit on the adaxial surfaces of the top leaves, but neither group of workers provided data on the morphology of the reproductive structures. Nor did they prove an association between the “white deposit” and the ascostromata of *B. sclerotica*. Govinda and Thirumalachar (1963) designated the variety *deformans* for *B. sclerotica* on two *Cymbopogon* species, the variety being based solely on differences in symptomatology.

Janardhanan et al. (1991) stated that the ascostromata of *B. sclerotica* were arranged on hypothalli (sic) over the nodes of the inflorescences of *Cymbopogon flexuosus* (Staud.) Wats. Their interpretation of the relationship of the ascostromata with host organs is incorrect. Like workers before them, they found an *Ephelis* fructification occurring as “sticky white powdery striae” on the top unfolding leaves, but did not prove that it was the anamorph of *B. sclerotica*.

Srinivasan and Thirumalachar (1969) studied *B. sclerotica* in axenic culture. They noted that in cultures derived from ascospores, conidia developed in whorls at the apices of short lateral “branches.” The conidia differed from those produced on the upper leaves, being slightly broader at the base and “not truly acicular.” In axenic culture, colonies which developed from “epheloid” conidia transferred from the host were identical with those derived from ascospores. Although no data on spore morphology were provided, their illustration indicate that ascospores were 88–115 \( \mu \text{m} \) long and 3-septate, and conidia were 17–20 \( \mu \text{m} \) long and broadly acicular.

White and Reddy (1998) erected *Parepichloe*, with *P. cinerea* (Berk. & Br.) White & Reddy as the type species. The new genus was separated from *Epichloe* primarily on the basis of its epibiotic habit, black ascostromata (compared to the endophytic habit and light yellow-orange color of ascostromata of *Epichloe*) and their occurrence on season grasses. No anamorph was described for the type or any other species. They also transferred *Balansia cynodontis* Syd., *Epichloe bambusae* Pat., *Epichloe oplismeni* P. Henn., *Epichloe sasae* Hara, *Epichloe sclerotica* Pat., and *Epichloe volkensii* P. Henn. to the new genus. Two distinct groups were recognized, the first containing only *cinerea* whose stromata do not contain leaves of the host, and the remainder whose ascostromata contain leaves. Species in the latter group were separated on the basis of the length of the stroma, and of the “stromal leaf blade,” presumably the protophyll.

The transfer of some of the species other than *cinerea* to *Parepichloe* is questionable. First, for some species transferred to the genus the ascostromata
surround the underdeveloped inflorescence, while for others they surround the leaves of the axillary buds. Second, no anamorph were described for Parepichloë. Finally, the ascus tip of _P. cinerea_ was described as flattened, while for the other species the tip was rounded. White and Reddy (1998) recognized that the ascostromata of _B. cynodontis_ were similar to _E. sclerotica_ and that they were different to the stromata of species of _Balansia_ subsp. _Eubalansia_ which form on inflorescences, those of species of _Balansia_ subsp. _Dothichloe_ which form on the surfaces of leaves, and ascostromata of epibiotic _Balansia_ species which are pulvinate or stipitate.

### 3.2. Symptomatology

For all six grasses studied in southern Queensland, healthy plants flowered during summer and consisted of short, vegetative tillers and taller tillers bearing inflorescences. Flowering tillers of some of the hosts were commonly branched ( _P. criniforme_ and _E. stricta_), while the others were rarely branching. Over the summer months _Nigroconurus_-infected tussocks consisted of three tiller types: (1) predominantly vegetative tillers bearing black, curved ascostromata of _N. scleroticus_ at the nodes, and on the upper leaves an effuse white fungal mass on which the anamorph of an _Ephelis_ species developed, (2) short, vegetative tillers, and (3) rarely, flowering tillers. For all hosts, normal flowering tillers rarely developed on tussocks which produced _Nigroconurus_-infected tillers, and when they did there were no signs of infection on the flowering tillers.

Diseased tussocks of five of the hosts ( _C. refractus_, _E. stricta_, _P. criniforme_, _P. scrobiculatum_, and _S. leiocladum_) had lower profiles than healthy tussocks, because infected tillers were shorter than flowering tillers. Doidge (1948), Govindu and Thiramalchar (1963), and Viennet-Bourgin (1964) reported a similar decrease in the height of tillers of some grasses infected by fungi considered in this chapter to be synonymous with _N. scleroticus_. Luttrell and Bacon (1982) and Clay and Jones (1984) noted a similar change for tillers of hosts infected by _A. hypoxylon_ and _M. atramentosa_. For the other Queensland host ( _T. triandra_), infected tillers were longer than flowering tillers, as were tillers of _Cynodon dactylon_ (L.) Pers. infected by a fungus with features similar to _N. scleroticus_ (King, 1918). Govindu and Thiramalchar (1963) also noted such differences between grasses infected by _B. sclerotica_.

Infected tillers on all of the hosts had significantly more nodes and were thinner than flowering tillers. For example, _N. scleroticus_-infected tillers of _S. leiocladum_ had 5–20 nodes, while flowering tillers had 2–4 nodes, and for _T. triandra_ infected tillers had 11–25 nodes while flowering tillers had 6–10 nodes. On the Queensland hosts of _N. scleroticus_, the closely placed nodes, together with a tendency for short, stiff leaves in the upper parts of the infected tillers, led to the development of a “witch’s broom” effect. The production of
many axillary shoots on infected tillers of some of the grasses contributed further to the effect. Witch’s broom symptoms have been recorded for \textit{B. sclerotica} on \textit{Cymbopogon flexuosus} [Steud.] Wats. (Janardhanan et al., 1991), \textit{B. pilulaeformis} (Diehl, 1950), and \textit{B. gaduae} (Möller, 1901).

Nearly all \textit{Nigrocornus}-infected tillers seen on grasses in Queensland were vegetative. Occasionally, rudimentary inflorescences characterized by an abnormal arrangement of the floral elements and the lack of caryopses developed on \textit{Nigrocornus}-infected tillers of \textit{S. leiocladum}, \textit{P. criniforme}, \textit{E. stricta}, and \textit{P. scrobiculatum}. King (1918) reported that when inflorescences developed on tillers infected by a \textit{Nigrocornus}-like fungus they were distorted, while Govindu and Thirumalachar (1963) reported “sterile inflorescences” on infected tillers. Undeveloped (or aborted) inflorescences have also been reported for \textit{A. hypoxylon} (Leuchtmann and Clay, 1989), and \textit{B cyperi} (Clay, 1986a).

Three types of abnormalities of \textit{Nigrocornus}-infected tillers on \textit{S. leiocladum} were observed. The first consisted of a twisting and coiling of the upper portions of tillers protruding through the overlapping margins of the surrounding leaf sheath. The second consisted of a short section of culm with closely placed nodes at the top of an infected tiller. A short shoot, and roots, developed from each of the nodes. The third type of abnormality consisted of looping of the upper 2–4 leaves, due to the adherence of the tip portions in the gelatinous hyphal matrix associated with the anamorph of \textit{N. scleroticus} on the leaf below. “Tangle top,” similar to the last abnormality described above, has been reported for \textit{M. atramentosa} (Luttrel and Bacon, 1977).

Some of the changes to tillers brought about by infection by \textit{N. scleroticus} are common to many of its hosts. It is possible that changes in the growth regulators in diseased tussocks, as reflected in the morphology of infected and flowering tillers, may have occurred. Rykard et al. (1985) hypothesized that infection of hosts by \textit{M. atramentosa} may change the hormonal balance of the plants, suppressing the development of inflorescences, and Clay (1986b) surmized that vivapary in inflorescences of \textit{Cyperus virens} Mich. infected by \textit{B. cyperi} was caused by hormonal imbalances. Porter et al. (1985) reported that \textit{B. epichloë} produced growth regulators in culture, and it is highly likely that other balansoid fungi do. Despite these common changes, the growth characteristics of a particular host has an influence on the symptoms displayed by infected tillers. Such differences in morphology and development between hosts has not always been appreciated. Govindu and Thirumalachar (1963) erected \textit{Balansia sclerotica} var. \textit{deformans}, based solely on differences in symptomatology of two hosts.

### 3.3. Survival

There is compelling evidence that \textit{N. scleroticus} and other balansoid fungi survive on tiller buds in the subterranean parts of the plants. Tussocks of
Queensland grasses produced *Nigrocornus*-infected tillers year after year, even when they were cut back to a short stubble over winter. Studies on infected plants of *S. leiocladum* and *P. criniforme* over several years have shown that hyphae of *N. scleroticus* are systemic and epiphytic. During winter, when both grasses are dormant, hyphae can be found surrounding the meristems in tiller buds on the subterranean tiller bases of tussocks. Hyphae of the epiphytic species *A. hypoxylon* and *A. texensis* were also found proximal to the meristems of tiller buds and later on the surfaces of meristematic regions in young tillers (Leuchtmann and Clay, 1988). Clay and Frenz (1993) reported that *B. pilulaeformis* systemically infected *Chasmanthium laxum* (L.) Yates when new meristems and tiller primordial were colonized from preexisting infected tillers. Survival in tiller bases was hypothesized for *B. aristidae* on *Aristida purpurascens* (Phelps et al., 1993), and *M. atramentosa* on two hosts (Rykard et al., 1985).

Epiphytic hyphae were also found on the tiller abnormality consisting of closely placed nodes described in the previous symptomatology section above. It is possible that such structures could develop into new *Nigrocornus*-infected plants under the right conditions. Janardhanan et al. (1991) claimed that 4% of seeds from inflorescences of lemongrass inoculated with *B. sclerotica* produced infected plants, raising the possibility of another mode of survival.

### 3.4. Anamorph Development

After activation of tiller buds, the first leaf of the tiller elongates and envelops the apical meristem and embryonic leaves in a tight longitudinal roll. Contemporaneously, the hyphae of *N. scleroticus* associated with the growing point increase in mass, keeping the space between the growing point and tightly rolled first leaf completely filled. The hyphae are immersed in a gelatinous substance which is not miscible with lactophenol. As the first leaf elongates, the second leaf of the growing point expands and grows upward within the tightly rolled first leaf. Hyphae immersed in the gelatinous matrix fill the space inside the roll.

When the first-formed leaf reaches a length of 5–10 cm it begins to unroll, from the tip toward the base. Just prior to the unrolling of the leaf blade, conidiophores develop on the vegetative hyphae beneath the leaf roll. The second leaf elongates upward through the surrounding leaf sheath of the first-formed leaf. Soon after the tip of the second leaf emerges past the ligule of the first leaf, the hyphae and gelatinous substance on the outer surface of the second leaf begin to dry out rapidly, while the hyphae within the rolled leaf remains immersed in the gelatinous substance. The just-emerged leaf elongates while still tightly rolled, a condition aided by the binding action of the hyphal matrix, which can cover the entire adaxial surface of the rolled blade. As the blade unrolls,
the hyphal matrix shrinks and dehydrates. The spatial and temporal relationships between hyphae and host tissue in the growing point and on each new leaf of infected tillers are identical with those outlined above. The hyphae remain epiphytic on all the leaves during the elongation of infected tillers.

The ontogeny of conidiophores and conidia was followed using material collected from the upper portions of infected tillers and from observations of the fungus growing on yeast peptone agar in slide cultures. The cultures had been established from single conidia which had been streaked onto the surface of water agar. The slide cultures were incubated in the dark at 25 ± 2°C for 5 days, then examined at hourly intervals. The development of individual conidiophores and conidia could be followed for more than 20 h.

The conidiophores are first evident as small swellings close to the septa of the vegetative hyphae. They elongate rapidly perpendicular to the hyphae. By the time the first conidium is evident, the conidiophores are hyaline, thin-walled, predominantly simple but occasionally branched, aseptate, 2–3 μm wide, and up to 40 μm long. A conidium develops holoblastically at the apex of the conidiophore as an ovate swelling 3–4 μm long and 2–3 μm wide. The conidium elongates rapidly. When it is 10–18 μm long, the conidium is delimited by a septum that develops between its base and the apex of the conidiophore. Just before the first conidium reaches its final length, a second conidium develops to one side of and just below it. As the second conidium expands, the first conidium detaches from the conidiophore and is pushed to one side by the second conidium. When fully developed, conidia are 16–24 μm long and up to 1.5 μm wide. Additional conidia develop in a similar pattern. During the period of conidium formation, the conidiophores elongate only a very short distance. The passively liberated conidia congregate about the apex of the conidiophore, remaining in that position for some time (Fig. 1A).

The asexual fructifications on leaves of tillers of the six grasses bearing ascostromata of *N. scleroticus* have the following characteristics: vegetative hyphae epiphytic, hyaline, thin-walled, septate, branched, 2–3 μm wide, aggregated into effuse sheets 5–10 μm thick covering the adaxial leaf surfaces and breaking up into fragments when dry; conidiophores arising near septa of the vegetative hyphae, and growing almost at right angles to the long axes of the hyphae, simple or very occasionally with one or two branches, aseptate, up to 40 μm long, 2–3 μm wide; conidia produced holoblastically at the apex of the conidiophore, hyaline, aseptate, multiguttulate, narrowly obclavate, 13–29 μm long and 1.0–1.5 μm wide at a point one-sixth to one-quarter of the distance from the base to the tip, then tapering gradually to a narrowly rounded tip.

In my opinion, the effuse conidial fructifications are those of a species of *Ephelis*. The ontogeny and morphology of conidia *N. scleroticus* are identical to those of the *Ephelis* anamorphs of several balansioioid fungi (Rykaerd et al., 1982, 1984; Morgan-Jones and White, 1989, 1992). *Ephelis* appears to be a highly
variable genus, with some species having effuse fructifications on leaves, others having compound conidiomata on stromata, and yet others which produce both on the same tiller. Clearly, further taxonomic studies are needed on this genus. *Ephelis* needs to be redescribed to accommodate the pleomorphism of the reproductive structures.

### 3.5. Teleomorph Development and Morphology

On infected tillers of all six hosts, axillary buds rarely develop in the axils of the first two or three leaves. Later-formed axillary buds are enveloped by hyphae in the early stages of their development at the tiller’s growing point, and they do not elongate into branches. The first leaf of an axillary bud, the protophyll, elongates rapidly and encloses the bud. The hyphae associated with the bud expand and fill the space between the protophyll and the bud’s growing point to form a compact stroma (Fig. 2). They hyphal mass increases in bulk as the axillary bud grows, at first beneath the enclosing protophyll, but later bursting through it. The stroma expands for some time beneath the surrounding leaf sheath but then pushes through the overlapped edges of the surrounding leaf sheath and continues its growth, ultimately becoming black and papillate and either curved or straight, depending on the host. During its development the base of the stroma remains adherent to the protophyll, so the curvature of some ascostromata is probably due to the differential expansion of hyphal tissue, with the expansion being restricted on one side by the adherence of the protophyll to the ascostroma. On any infected tiller there are ascostromata at various stages of development, the most mature
being at the lowermost nodes. At maturity, ascostomata have three layers: an inner cortex composed of hyaline, thin-walled hyphae; an outer cortex consisting of compact, dark, septate hyphae; and an outer rind of tightly compacted, pigmented, closely septate hyphae. The hyphae remain epiphytic during the development of the ascostromata, and at maturity.

Perithicum initiation starts just before the developing ascostromata bursts through the surrounding leaf sheaths, near the base of the outer cortex. The subsequent development of perithecia is identical to that described for *A. hypoxylon* (Leuhtmann and Clay, 1989), *A. texensis* (Morgan-Jones et al., 1993), *B. aristidae* (Morgan-Jones, 1993), *B. epichloë* (Morgan-Jones and Phelps, 1995), and *M. atramentosa* (Luttrell and Bacon, 1977).

Various morphological features of mature ascostromata of *N. scleroticus* on the six hosts are presented in Table 1. The ascostromata dimensions vary considerably between hosts, from 3–5 mm × 1.0–1.5 mm on *T. triandra*, to 10–26 mm × 2–3 mm on *S. leiocladum*. On *C. refractus, E. stricta, S. leiocladum*, and *T. triandra*, the entire surface of ascostromata was papillate.
<table>
<thead>
<tr>
<th>Host</th>
<th>Ascostromata dimensions (mm)</th>
<th>Perithecia dimensions (μm)</th>
<th>Asci dimensions (μm)</th>
<th>Ascospores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cymbopogon refractus</td>
<td>5–8 × 1.0–2.0</td>
<td>490–540 × 105–180</td>
<td>168–242 × 5.0–7.0</td>
<td>144–200</td>
</tr>
<tr>
<td>Entolasia stricta</td>
<td>4–8 × 1.0–1.5</td>
<td>230–297 × 72–108</td>
<td>170–210 × 3.0–5.0</td>
<td>134–182</td>
</tr>
<tr>
<td>Paspalidium criniforme</td>
<td>3–7 × 0.5–1.0</td>
<td>266–386 × 93–133</td>
<td>152–195 × 4.5–6.0</td>
<td>146–180</td>
</tr>
<tr>
<td>Sorghum leiocladum</td>
<td>10–26 × 2.0–3.0</td>
<td>364–480 × 130–182</td>
<td>158–237 × 3.0–5.0</td>
<td>134–210</td>
</tr>
<tr>
<td>Themeda triandra</td>
<td>3–5 × 1.0–1.5</td>
<td>290–300 × 120–175</td>
<td>164–186 × 5.0–7.0</td>
<td>154–182</td>
</tr>
</tbody>
</table>
while on a few ascostromata of *P. criniforme* there were discrete, raised papillate areas separated by nonpapillate areas. Although some ascostromata on infected tillers of *P. scrobiculatum* were arched, black, and up to 30 mm long, none ever became papillate, and no perithecia ever developed. The perithecia, although variable in dimensions, were obpyriform-lageniform, with a distinct wall 6–15 μm thick consisting of laterally compressed hypha and a tapering neck lined with periphyses. Ascii, which developed from an ascogenous zone in the base of the perithecia, were cylindrical, unitunicate, thin-walled, of variable length, and with a hemispherical cap 2–3 μm in diameter and perforated by a fine pore 1.0–1.5 μm wide (Fig. 1B). The asci are at first separated by fine paraphyses, which disintegrate during ascus development. Eight filiform ascospores develop in each ascus. The ascus morphology of *N. scleroticus* is identical to species of *Balansia* and *Atkinsonella* (Diehl, 1950; Jones and Clay, 1987).

Data on mature ascospores were gathered from those which had been ejected onto water agar from mature ascostromata. Freshly ejected ascospores were 7-septate, with one thick central septum, and three thin septa on each side of the centre one. After 4–8 h, the ascospores assume a zigzag shape, and one germ tube, 1.0–1.5 μm wide, grows in a hooked manner from each cell of the ascospore at a point very near to a septum. Approximately 12 h after germination, ascospores break into two 3-septate parts, approximately equal in length. A similar mode of ascospore germination was reported for *A. hypoxylon* and *A. texensis* (Leuchtmann and Clay, 1989), *B. sclerotica* (Srinivasan and Thirumalachar, 1969), and *Balansia* sp. (characteristics identical to *N. scleroticus*) (King, 1918). For *N. scleroticus*, hyphae which develop from the germ tubes are hyaline, thin-walled, septate, branched, and 1.5–3.0 μm wide (Fig. 1C). Between 24 and 48 h after germination, conidiophores grow as short lateral branches from the hyphae. The mode of conidiophore and conidium ontogeny has been described previously. These features of ascospore germination contrast with species belonging in subgenus *Dothichloë*, which according to Phelps and Morgan-Jones (1993) and White et al. (1996, 1997) disarticulate into either four or eight 1-septate part-ascospores before ejection from the ascus. For *M. atramentosa*, ascospores separate into fusoid, aseptate part-ascospores before discharge from the ascus, and a single, curled germ tube develops from each part-ascospore (Luttrell and Bacon, 1982; Phelps and Morgan-Jones, 1993).

### 3.6. In-Vitro Studies

Evidence for a genetic relationship between ascostromata of *N. scleroticus* and the foliar conidial fructifications on the adaxial surfaces of uppermost leaves of tillers bearing ascostromata was gathered by comparing colonies derived from single ascospores with those from single conidia. Two hosts, *S. leiocladum* and
were used in the studies, and the colonies were grown on yeast peptone agar for 28 days at 25°C in a dark incubator.

For both hosts, colonies derived from single part-ascospores were identical with those derived from single conidia. There were no significant differences ($P \leq 0.05$) in colony diameter or conidium length between colonies derived from both spore types for either host. All colonies were white, soft, and with abundant surface and submerged mycelium. However, there were differences in colony morphology between isolates of the two hosts (Table 2), particularly in the margin, mycelium form, and color changes in the medium. Conidia from all cultures were very similar in all respects to those found on infected tillers (Table 2).

The data presented in this section have established a genetic relationship between the ascal state of *N. sclerotica* and the foliar fructifications on *Nigrocorpus*-infected tillers for two of its hosts. It is highly probable that a similar relationship exists for all of its hosts. Although Srinivasan and Thirumalachar (1969) stated that colonies derived from “germinating ephelidial conidia” were identical with “that obtained from germinating conidia in all respects” this is the first report to provide definitive proof.

### 3.7. Herbarium Specimens—Type Specimen

A request for the type specimen of *Epichloë sclerotica* was made to Farlow Herbarium (FH), which houses Patouillard’s herbarium. The specimen forwarded was the only one of that name deposited in the herbarium (Cacavio, personal communication). On the specimen packet, the following annotation is attached: “This is an authentic collection made one month after the supposed collection of the holotype.” The annotation was placed there by D. H. Pfister while working on his “Annotated Index to the Fungi described by N. Patouillard” (Pfister, 1977) (Cacavio, personal communication). Until evidence to the contrary is provided, this specimen is designated as the Neotype of *Balansia sclerotica*, in accordance with Article 7 of the International Code of Botanical Nomenclature.

The type specimen of *B. sclerotica* consists of the upper portions of four culms each displaying a witch’s broom effect, there being many short, closely arranged axillary shoots from the culms. At each node of the short branches, a black, curved, papillate ascostroma 2.0–4.5 mm × 0.5–1.2 mm surrounds the axillary bud. The protophyll of the axillary bud is adherent to the ascostroma for its entire length, extending for 0.5–1.0 mm beyond the distal end of the ascostroma. The lageniform perithecia are 180–250 μm long with a subglobose-oval basal part 69–105 μm wide, tapering to a short neck which constitutes 20–30% of the total length of the perithecium. The neck of the perithecium is 25–50 μm wide, opening to the outside where the surface of the stroma is papillate. The ostiolar canal is lined with filiform periphyses 5–10 μm long and
<table>
<thead>
<tr>
<th>Colony characteristics</th>
<th>Sorghum leiocladum</th>
<th>Paspalidium criniforme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony Margin</td>
<td>Entire</td>
<td>Irregular</td>
</tr>
<tr>
<td>Surface</td>
<td>Slight raised</td>
<td>Umbonate in center, closely appressed elsewhere</td>
</tr>
<tr>
<td>Context</td>
<td>Cottony</td>
<td>Compact in center, loose elsewhere</td>
</tr>
<tr>
<td>Medium color change</td>
<td>Dark livid in center</td>
<td>Vinaceous buff in center</td>
</tr>
<tr>
<td>(Rayner, 1970)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascospore derived</td>
<td>47 – (50.8) – 54</td>
<td>40 – (41.6) – 43</td>
</tr>
<tr>
<td>Conidium derived</td>
<td>46 – (48.5) – 52</td>
<td>39 – (40.8) – 43</td>
</tr>
<tr>
<td>Conidium length (μm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascospore derived</td>
<td>11 – (18.6) – 25</td>
<td>13 – (18.3) – 25</td>
</tr>
<tr>
<td>Conidium derived</td>
<td>13 – (18.4) – 25</td>
<td>11 – (18.5) – 26</td>
</tr>
</tbody>
</table>
0.5–1.0 \mu m in diameter. Ascii are 150–240 \mu m long and 3–7 \mu m wide, unitunicate, and with a thin wall except at the apex, where there is a distinct apical cap, 3–4 \mu m in diameter and perforated by a narrow pore. The ascospores are hyaline, filiform, 7-septate, 140–220 \mu m long and 1.0–1.5 \mu m wide.

On the adaxial surfaces of the upper leaves, there are white flakes composed of thin-walled, septate, branched hyphae aggregated into thin sheets. Conidia found on the hyphae sheets were hyaline, aseptate, guttulate, narrowly obclavate, and 14–22 \mu m \times 1.0–1.5 \mu m. The conidia were widest at a point one-sixth to one-fourth the distance from the base to the tip, then gradually decreasing in width to a narrowly rounded tip. They were identical with those found on *Nigrocornus*-infected tillers on living plants of six Queensland grasses.

In his original description of *Epichloë sclerotica* (sic), Patouillard (1890) did not identify the host, and no inflorescences were found on any of the culms in the type specimen. Based on leaf anatomy, the grass belongs in the Tribe Andropogoneae and is very similar to that of species of *Cymbopogon* (Watson, personal communication). Until evidence to the contrary is gathered, the host of the type specimen of *Balansia sclerotica* should be considered as a species of *Cymbopogon*.

### 3.8. Herbarium Specimens—Other Specimens

Over 100 specimens deposited as *Balansia sp.*, *Balansia sclerotica*, or fungi considered to be synonymous with *N. scleroticus* in herbaria in Australia and overseas were examined. On these specimens the ascostromata were black, corniform, curved or straight, adnate with the protophyll, and variable in length and width (Fig. 3). At one extreme were ascostromata on *T. triandra* (IMI 74093), which measured 15–17 mm \times 3–4 mm, and at the other were those on *Oplismenus hirtellus* (L.) Beauv. (IMI 4607), which were 2–3 mm \times 1.0–1.5 mm. For most specimens the length:width ratio of ascostromata was \approx 2:1, but on the specimens of *Triodia* species collected in Australia (BRIP 2818, BRIP 13406), the ratio was very close to 1:1. The type specimen of *Balansia axillaris* (Cooke) Petch (K. ex. Herb. F. M. Bailey 897, 898) consists of two different grasses and in specimen number 898, the protophyll is not adnate to the ascostroma. On the ascostromata in the type specimen of *Balansia cynodontis* Syd. (PRE 23473) there are several papillate portions separated by nonpapillate areas. A similar condition is found on some ascostromata of *N. scleroticus* on *Paspalidium criniforme* collected in southern Queensland.

The characteristics of the perithecia, asci, and ascospores on ascostromata which could be examined were in most cases identical to those of the corresponding characteristics of the type specimen. For three specimens (IMI 4367, IMI 114417, IMI 22213), the range of ascospore length (160–280 \mu m) was different from that of the type specimen (140–220 \mu m). The apical thickening of
asci of *Balansia trachypogonis* Doidge is not hemispherical as in *N. scleroticus*, but is in the form of slight thickening of the ascus wall, similar to that described for *M. atramentosa*. A dried hyphal matrix was found covering the abaxial surfaces of the uppermost leaves on approximately 35% of the specimens, including the type specimens of *Balansia cynodontis* Syd. (PRE 23473), *Balansiopsis schumanniana* (P.Henn.) Höhnel (B), *Balansia trachypogonis* Doidge (PRE 9543b), and *Ophiothidis paspali* P.Henn. (S). The conidia in these matrices were identical in all respects to those found on the type specimen. On most of the specimens which lack a hyphal matrix on leaves, the upper portions of the tillers are missing.

3.9. Taxonomy—Description of *Nigrocornus Scleroticus*

The specimens of *Nigrocornus scleroticus* and of species considered to be synonyms of that species have a number of features in common. The ascostromata are black, corniform, and surrounding the axillary buds at the nodes of culms. The considerable variation in the dimensions of the ascostromata on different hosts is probably a reflection of the morphology and development of the grass hosts. White and Reddy (1998) separated the species *cynodontis*, *oplismeni*, *sclerotica*, and *volkensii* (which they included in *Parepichloë*) on the lengths of the ascostromata and the “basal stroma leaf” (presumably the protophyll). Based

**Figure 3** Ascostromata of *Nigrocornus scleroticus* on three hosts. A, *Sorghum leiocladum* (Hack.) Hubb.; B, *Paspalidium criniforme* S.T. Blake; C, *Tridodia epactia* S.W.L. Jacobs. Bar = 10 mm.
on the research reported here, there is no justification for separating the species. These authors also reported that based on rDNA sequence data, the species schumanniana and sclerotica are identical.

The morphology of the anamorph and teleomorph of N. scleroticus on the living hosts and on herbarium specimens are identical in most cases. The notable exceptions are the apical thickening of asci of B. trachypogonis, and the discrete papillate areas on ascostromata of B. cynodontis and of N. scleroticus on Paspalidium criniforme. Further morphological and molecular studies are needed to evaluate the relationships between these fungi.

The association between the effuse Ephelis fructifications on the upper leaves and the ascostromata at the nodes on tillers of some living grasses has been conclusively proved. The presence of the effuse fructifications on the type and many other herbarium specimens indicates that the anamorph of N. scleroticus is common, while the absence of the Ephelis fructification on other herbarium specimens is an indication of its evanescent nature.

The morphology and location of the ascostromata and the effuse Ephelis fructifications which are spatially separated from the ascostromata distinguish Nigrocornus scleroticus from all other calvicipitalean fungi. However, the new genus has characteristics which align it with Atkinsonella, Balansia, and Myriogenospora, namely, black, papillate ascostromata, and similar development and morphology of perithecia, asci, ascospores, and conidia. In addition, Kuldau et al. (1997) demonstrated that B. sclerotica (sic) was in the Ephelis clade, together with species of Balansia, Myriogenospora and Atkinsonella.

The genus Nigrocornus is formally described below.

3.9.1. Nigrocornus Ryley & Langdon gen. nov

Ascostromata includentia gemmas axillares in nodis culmorum, dimensiones variabiles, nigra, cornuta, papillata; perithecia immersa, lageniformia vel obpyriformia, ostiolo protrudenti, paraphyses nullae; asci cylindrici operculo hemisphaericio, apicall; ascosporae octo in quoque asco, filiformes, hyalinae, 7-septatae demum factae 3-septatae; conidiophora simplicia interdum ramificata, indeterminata, ex hyphis exorientibas, hyphae in tegentes foliorum superficies adaxiales, facientes stratum effusum; conidia holoblastica, anguste obclavata, aseptata, multitubulata.

3.9.2. Sp. typ. N. scleroticus

Ascostromata enclosing axillarys buds at the nodes of culms, dimensions variable, black, corniform, papillate; perithecia immersed, lageniform to obpyriform, with a protruding ostiole, no paraphyses, asci cylindrical with a hemispherical apical cap; ascospores in each ascus, filiform, hyaline, 7-septate, ultimately becoming 3-septate; conidiophores simple, occasionally branched,
indeterminate, arising from hyphae, hyphae covering adaxial leaf surfaces as an effuse layer; conidia holoblastic, narrowly obclavate, aseptate, multiguttulate.

3.9.3. *Nigrocornus scleroticus* (Pat.) Ryley comb. nov

Basionym: *Epichloë sclerotica* Pat., *J. De Bot.* 4:65 (1890)

Synonyms:


*Ophiodothis oplismeni* (P. Henn.) P. Henn., *Annln naturh. Mus. Wien* 15: 2 (1900).


*Balansia triraphidis* (name only).

*Ophiodothis paspali* P. Henn. (name only).

Ascostromata occurring singly at nodes of culms and enclosing axillary buds, dimensions variable, black, corniform, papillate, closely associated with prophyll of axillary bud, internally three distinct zones, rind, outer cortex, and inner cortex; perithecia immersed in periphery of ascostromata, lageniform to obpyriform, dimensions variable, no paraphyses; asci cylindrical, with a hemispherical apical cap, dimensions variable; ascospores eight in each ascus, 140–220 × 1.0–1.5 μm (120–210 × 1.0–1.5 μm in other specimens), filiform, hyaline, 7-septate, breaking into two almost equal 3-septate parts after germination; conidiophores simple, occasionally branched, indeterminate, up to 40 μm long, 2–3 μm wide, arising from hyphae which cover the adaxial leaf surfaces as an effuse layer, conidia holoblastic, 14–22 × 1.0–1.5 μm.
(13–29 × 1.0–1.5 μm in other specimens), narrowly obclavate, aseptate, multiguttulate.

Type Specimen: On *Cymbopogon* Sp.?, Fac-Bin, Tonkin (Vietnam), 11.xi.1887, Balansa (Neotype) (FH).

### 3.10. Host Specificity and Distribution

*Nigrocornus scleroticus* has been recorded on 29 grass genera (Table 3). Using the database of Watson and Dallwitz (1992 onwards), over 70% (23) of these belong in the subfamily Panicoideae, with 13 in the tribe Andropogoneae, 10 in the Paniceae, and one in the Arundinelleae. Four belong in the subfamily Chloridoideae (main chloridoid assemblage), and the other one has been assigned to the subfamily Arundinoideae. These grass genera are predominately tropical and subtropical (40°N to 40°S) in distribution (Watson and Dallwitz, 1992 onwards) and most have a C₄ photosynthetic pathway. Kellogg (2001), in her summary of the current knowledge on evolutionary development of the grasses, found that grasses in the Panicoideae and Chloridoideae are very closely related, and belong in the PACC group (panicoids, arundinoids, chlorioids, and centothecoids).

In the opinion of Hsiao et al. (1999), the early grasses evolved during the mid-Upper Cretaceous in or near the tropical forests of South America, where they remained in low numbers. Archetypes of all the major subfamilies probably evolved after rapid climate change in the Upper Cretaceous in response to drastic climate change, and adaptive radiation subsequently set subfamilies apart. The Arundinoideae developed in the more seasonal open savannah/semiarid regions, while the Panicoideae and Chloridoideae evolved rapidly and developed and adapted

<table>
<thead>
<tr>
<th><strong>TABLE 3</strong> Grass Host Genera of <em>N. scleroticus</em></th>
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</thead>
<tbody>
<tr>
<td><strong>Subfamily Panicoideae</strong></td>
</tr>
<tr>
<td>Tribe Andropogoneae: <em>Anadelphia, Andropogon, Bothriochloa,</em></td>
</tr>
<tr>
<td><em>Chrysopogon, Cymbopogon, Dichanthium, Hyparrhenia, Ischaemum,</em></td>
</tr>
<tr>
<td><em>Poganthemum, Schizachyrium, Sorghum, Themeda, Trachypogon</em></td>
</tr>
<tr>
<td>Tribe Paniceae: <em>Anthephora, Brachiaria, Digitaria, Entolasia, Oplismenus,</em></td>
</tr>
<tr>
<td><em>Panicum, Paspalidium, Paspalum, Poecilostachys, Setaria</em></td>
</tr>
<tr>
<td>Tribe Arundinelleae: <em>Arundinella</em></td>
</tr>
<tr>
<td><strong>Subfamily Arundinoideae</strong></td>
</tr>
<tr>
<td>Tribe Eriachneae: <em>Eriachne</em></td>
</tr>
<tr>
<td><strong>Subfamily Chloridoideae</strong></td>
</tr>
<tr>
<td>Main chloridoid assemblage: <em>Cynodon, Eragrostiella, Triodia, Triraphis</em></td>
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to tropical and subtropical climates where the more efficient C₄ carboxylation developed.

All of the specimens of *N. scleroticus* were collected in the tropics and subtropics in Africa, India, southeast Asia, and Australia. *Nigrocornus* is absent from the Americas, where there is a rich balansioid flora. The global distribution of *N. scleroticus* reflects a long association with its hosts.

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Processes of Species Evolution in *Epichloë/Neotyphodium* Endophytes of Grasses

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1. INTRODUCTION

Members of the fungal family, Clavicipitaceae, are biotrophic symbionts (including parasites and mutualists) of a variety of host organisms including plants, other fungi, and invertebrate animals. Most plant-associated members of this family cause diseases on monocots, though such diseases are unusual in several regards. First, only small and defined regions, or specific organs, of the plant are affected. For example, *Claviceps* spp. replace ovaries of the florets and produce their own resting structures (sclerotia, also called ergots) in place of the seeds that would otherwise have developed there. Other Clavicipitaceae are capable of very long-lived associations with plants, and grow systemically throughout much of the above-ground host tissue, but these fungi also cause disease symptoms and signs only on a small portion of otherwise healthy leaves, or in association with the inflorescences or florets. The limited diseases and some host benefits, such as gigantism and antiherbivory, indicate that plant symbioses with Clavicipitaceae can range in a continuum from mildly antagonistic to
**FIGURE 1** Life cycles of *Epichloë festucae*, *E. typhina*, *Neotyphodium lolii*, and an *N. lolii × E. typhina* hybrid, and the evolutionary origin of *N. lolii* and the hybrid. Solid arrows indicate systemic growth and (in asexual life cycles)
mutualistic (Clay, 1990; Schardl and Clay, 1997). At the mutualistic extreme are symbioses with several of the Epichloë species, which are capable of completely asymptomatic symbiosis with their host grasses, and can even transmit vertically via seeds (Sampson, 1933). In the life cycles of some Epichloë species (Fig. 1) such vertical transmission predominates, and may obviate the need for horizontal (contagious) transmission.

Interestingly, vertical and horizontal transmission processes of Epichloë spp. actually antagonize each other (Fig. 1). Furthermore, vertical transmission entails asexual propagation of the fungus, whereas sexual spores (meiotically derived ascospores) normally mediate horizontal transmission (Brem and Leuchtmann, 1999; Chung and Schardl, 1997). In the most extreme cases, Epichloë lineages lose sexual expression altogether and only transmit vertically. Yet such asexual lineages cannot be assumed to be fully clonal, since other modes of genomic recombination may be available to them, namely, parasex (Caten, 1981; Glass and Kuldau, 1992) and hybridization (Fig. 1). Indeed, phylogenetic analysis indicates that interspecific hybridization is extremely common among these asexual Epichloë endophytes, as we discuss in this chapter.

The main focus of this chapter is on the asexual, vertically transmitted grass endophytes derived from the Epichloë spp. Due to botanical convention, asexual Epichloë lineages are classified as Neotyphodium spp. Being vertically transmitted only, the Neotyphodium spp. are completely dependent on individual host plants and their progeny for survival and dissemination. Such a situation is predicted to amplify selection for symbiont characteristics that enhance host fitness, resulting in mutualism (Ewald, 1987). However, considerable evidence indicates long-term costs of clonal propagation of organism or genomes (Bergstrom and Pritchard, 1998; Bidochka and De Koning, 2001; Butcher, 1995; Gordo and Charlesworth, 2000; Lynch, 1997; Moran, 1996; Muller, 1964; Rice, 1994), which may explain some of the alternative modes of recombination available to asexual fungi as well as the maintenance of sexual capabilities in the Epichloë species. In this chapter we present evidence from the endophyte system in support of this possibility, and pay particular attention to the preponderance of Neotyphodium spp. that have undergone interspecific hybridizations with Epichloë species.

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vertical transmission. Dashed arrows indicate spore transfers, including horizontal transmission of E. festucae (a), a transmission of E. festucae into Lolium perenne giving rise to the ancestor of N. loli (b), obligatory horizontal transmission of E. typhina on perennial ryegrass (c), and the infection by E. typhina of an L. perenne-N. loli symbiotum that was a prerequisite for interspecific hybridization (d).
2. RELATIONSHIP OF LIFE HISTORY AND EVOLUTIONARY TRAJECTORIES

Many have proposed that evolution trends from antagonism to mutualism. The problem with this proposition is that it is difficult to explain the persistent occurrence of antagonisms after so many eons of evolution. If, on the other hand, the trend were in the other direction, the converse problem would arise. It seems more reasonable to expect the balance of interactions between symbionts to evolve toward either mutualism or antagonism, depending on a variety of circumstances. Life history is an aspect that has received considerable attention in this regard, with emphasis on the interplay between transmissibility and virulence (Yamamura, 1993). As a rule, it appears that the most readily transmissible parasites tend to be the most antagonistic (virulent), and those that are poorly transmitted tend to be the least virulent. However, it is worth noting the wide gulf between attenuated virulence and mutualism; the evolutionary basis for the former is, at best, inadequate to account for the latter.

One symbiotic condition predicted to promote mutualism is vertical transmission (Ewald, 1987; Yamamura, 1993). Note, however, that this is not the only reason for mutualisms; for example, vertical transmission does not occur in legume–rhizobia mutualisms or mycorrhizas (Lewis, 1973). However, in the *Epichloë*/*Neotyphodium*–grass systems, relative tendencies to transmit vertically and horizontally can be assessed and the implications considered. In fact, the particular evolutionary processes acting on *Epichloë* and *Neotyphodium* spp. appear to have been greatly influenced by the prevailing transmission modes (Craven et al., 2001b). Insofar as evolution is governed by natural selection for enhanced fitness, these various evolutionary processes must reflect the selective forces that were brought to bear on the symbionts in the context of their various life histories.

Several *Epichloë* species, such as *E. festucae* (Leuchtmann et al., 1994), undergo dual life cycles on their hosts. One is an asexual life cycle that depends on host seed as the vehicle for dissemination (see top of Fig. 1). The other life cycle entails production of sexually derived ascospores that serve as contagious propagules, allowing infections of new plants or seeds that develop on previously uninfected plants. Interestingly, this sexual life cycle is exhibited only on flowering culms of the host, and once the fungus enters this stage the host cannot produce seeds on the same tiller. This is because expression of the fungal stroma surrounds, ramifies, and halts development of the immature inflorescence, hence the term “choke disease.” Other tillers exhibit normal development, though the endophyte is present in these asymptomatic tillers as well. Vertical transmission occurs via these asymptomatic tillers. The fungus grows in the floral meristems, the ovule, the developing seed, scutelum, and embryonic axis (Freeman, 1904;
Philipson and Christey, 1986; Sampson, 1933). Though there is sparse hyphal growth in the ovule and later in the embryo, the process is extremely efficient: all progeny seedlings possess the endophyte, and once the plants mature and flower they all exhibit precisely the same phenotype of sparse stroma development and highly efficient seed transmission. Which developmental program that *E. festucae* undertakes—either developing a stroma or transmitting in seeds—is determined on a tiller-by-tiller basis. Vertical transmission appears to be the prevalent mode of dissemination by *E. festucae*, and in many environments, *E. festucae* exhibits no stroma expression at all (Zabalgogeazcoa et al., 1999). Again, a strong tendency for vertical transmission is predicted to select for mutualism, and indeed *E. festucae* can be highly beneficial to host plants (Bazely et al., 1997; Saha et al., 1987) (but see Sampson, 1933).

Pleiotropic symbiosis exemplified by the dual transmission mode of *E. festucae* is shared by many strains of *E. amarillans*, *E. brachyelytri*, and *E. elymi* (Scharld and Leuchtmann, 1999; White, 1994). However, some strains, such as most *Epichloë typhina* isolates, only develop the stromal state and cannot be vertically transmitted (Chapter 6, this volume). Such “strong chokers” are typical of *E. baconii* on *Agrostis* and *Calamagrostis* spp., and *E. glyceriae* on *Glyceria striata* (Scharld and Leuchtmann, 1999; Scharld et al., 1997; White, 1993). These *Epichloë* species and strains that are only horizontally transmitted are considered the more antagonistic symbionts because they prevent whole plants from producing seeds, though they still may provide some considerable protective benefits to the host plants (Clay et al., 1993). The situations with *E. sylvatica* and *E. bromicola* are more complex, and the reader is referred to Chapter 6 of this volume. Finally, the asexual endophytes (*Neotyphodium* spp.) rely entirely on vertical transmission via seeds, and tend to be beneficial to their hosts (Brem and Leuchtmann, 2001; Clay, 1990), though such benefits are not necessarily universal for these types of interactions (Tibbets and Faeth, 1999; Wilson and Faeth, 2001).

Why might *Epichloë* evolution not tend toward mutualism in all cases? The crucial point is that all symbioses entail costs to the symbionts and hosts. It is likely that benefits outweigh the costs to at least one partner, so that in mutualisms both partners receive a net benefit. Indeed, costs are obvious in grass associations with *Epichloë/Neotyphodium*. For the host, some nutrition must be diverted for endophyte growth and propagation. For the endophyte, horizontal and vertical transmission antagonize each other: horizontal transmission requires stromata to form in a process that also prevents seeds from being produced on the affected tillers. Vertical transmission has the advantage of guaranteeing a home to the endophyte in subsequent generations (multiple years). However, without horizontal transmission the endophyte is locked into an individual host lineage. Endophytes that transmit both horizontally and vertically might be thought to have a special advantage, but the yield of contagious ascospores must necessarily
be dependent on the number of tillers with stromata, and their fitness depends on availability of compatible hosts to which they can spread (Craven et al., 2001b). Therefore, if the ecological circumstance is most favorable for endophytes that spread contagiously, the potential for vertical transmission may be reduced or even eliminated by selection. Conversely, whenever contagious spread is not adequately efficient, vertical transmission is more beneficial.

3. ESTABLISHING THE PHYLOGENETIC BACKBONE

Several phenetic and phylogenetic analyses (Glenn et al., 1996; Kuldau et al., 1997; Leuchtmann and Clay, 1990) have confirmed the previously proposed relationship of genus Neotyphodium (= Acremonium sect. Albo-lanosa) with genus Epichloe s. s. (Morgan-Jones and Gams, 1982; White and Morgan-Jones, 1987a). One can assume a priori that asexual lineages evolved from sexual lineages, since loss of the intricate developmental pathway for the sexual state is far more likely than its repeated invention (Schardl et al., 1991). Nevertheless, several questions can be asked as to the particulars of asexual endophyte evolution: How often have they arisen? Have they arisen from several Epichloe spp. or only very few? Have they arisen by simple loss of the sexual state or from more complex mechanisms? How long-lived are they relative to their ancestral sexual species? Are they ubiquitous, occurring in many hosts and places? To address these questions it is essential first to have information on Epichloe phylogeny that is as thorough as possible—namely, a rooted tree that encompasses the known diversity of this genus. The phylogenetic relationships of sexual Epichloe species are briefly presented here, and more extensively discussed in Chapter 6.

Three genes have been used together and separately to elucidate relationships among Epichloe species (Craven et al., 2001b). These are genes for beta-tubulin (tub2), translation elongation factor 1-alpha (tef1), and actin (act1), all of which are single-copy (no paralogs), and all shown in a genetic analysis of E. typhina to be unlinked from one another. Only the introns of these genes had sufficient variation for the intrageneric phylogeny, but major portions of the intron sequences could not be aligned with outgroup species such as Claviceps purpurea and Dussiella tuberiformis (= Echinodothis tuberiformis). When alignable regions from all three genes were combined, a root position was inferred with moderate support in bootstrap (Craven et al., 2001b; Chapter 6, this volume). Some additional evidence suggests the inferred root is correct. First, the root is close to the position of the midpoint root. Second, the root splits genus Epichloe into two clades with different patterns of host interactions and speciation. In one clade, designated the Epichloe main group, most biological species correspond to well-supported subclades and are associated with particular host species, genera, or tribes. In the other clade, designated the E. typhina
complex, interfertility relationships are complex, phylogenetic and biological species do not correspond well, and a single species (E. typhina) is genetically diverse and infects a broad range of hosts within subfamily Pooidae. In both clades, however, species associated with deeply rooted host tribes tend also to be deeply rooted. In fact, the phylogenetic pattern in the main clade is a close, though not absolute, match with that of the hosts (Scharl et al., 1997).

The relationships of many asexual endophytes (Neothyphodium spp.) with sexual Epichloë species are complex, as explained in the next section. Two of the Neothyphodium species are important to mention in this section because their relationships help fill out the phylogenetic backbone in Epichloë/Neothyphodium. One, Neothyphodium aotearoae (Moon et al., 2002), is associated with the Australasian grass, Echinopogon ovatus, and appears on a basal lineage in the E. typhina complex (Fig. 2). An undescribed species has a critical position in the Epichloë/Neothyphodium phylogeny, so here we will tentatively assign the name Neothyphodium “inebrians,” identifying a lineage that is among the more basal in the Epichloë main group. The placement of N. “inebrians” is approximately as expected in keeping with the tendency of Epichloë main-group species to track host phylogeny, since its host, Achnatherum inebrians, is in the deeply rooted tribe Stipeae.

In all, 11 clades are indicated, most corresponding to distinct Epichloë species (Fig. 2). Those species in well-supported clades are E. festucae, E. amarillans, E. glyceriae, E. elymi, E. bromicola, E. brachyelytri, N. “inebrians,” and N. aotearoae. Two clades of E. baconii are evident, one associated with Agrostis spp. hosts, and the other so far represented by a single isolate from Calamagrostis villosa. The two E. baconii clades never definitively group together in any gene tree, but both tend to group in a larger clade with E. amarillans and E. festucae. In the E. typhina complex, there is a poor relationship between described species and phylogenetic groups. Epichloë clarkii appears as a member of a clade that otherwise includes many of the E. typhina isolates, with which it is interfertile and appears to have recombined (Craven et al., 2001b). Epichloë sylvatica also appears interreticulated with E. typhina, even though E. sylvatica has exhibited very low or no interfertility in test matings with E. typhina. Interestingly, within the E. typhina complex is a subclade associated with host genus Brachypodium, consisting of E. sylvatica plus those E. typhina isolates from Bp. pinnatum (Chapter 6, this volume).

Host specificity is both characteristic of most Epichloë species and, as we discuss later, highly germane to the evolution of asexual endophytes. Among the stroma-forming isolates, almost every clade includes only those isolates from a certain host tribe, genus, or species (Craven et al., 2001b; Chapter 6, this volume, Fig. 2). Often the phylogenetic position of a new isolate of Epichloë can be predicted by knowledge of its mating compatibilities and the tribe of its host. In the Epichloë main group, isolates from host tribe Aveneae group in the largest
FIGURE 2  Backbone endophyte phylogeny based on combined tub2, tef1, and act1 intron sequences. Major clades are labeled above branches as a–k. Tree is midpoint rooted (left edge). Position of the root, using outgroups Claviceps purpurea and Dussiella tuberiformis, is indicated by arrow 1.

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clade, which includes *E. amarillans*, *E. festucae*, and *E. baconii*. Isolates from Poeae group with *E. festucae*, isolates from Triticeae with *E. elymi*, isolates from Bromeae with *E. bromicola*, isolates from Meliceae with *E. glyceriae*, and isolates from Brachyelytræae with *E. brachyelytri*. Even within the *E. typhina* complex there are distinct clades associated with some, but not all, host species: most notably *Poa nemoralis*, *Poa pratensis*, and *Brachypodium* spp. No stroma-forming isolate has been found that deviates from these relationships, but nonstromal (therefore, asexual) isolates often do. Two instances are noteworthy: an isolate from *Hordelymus europaeus* (Triticeae) grouped with *E. bromicola*, and an isolate from *Elymus virginicus* (Triticeae) grouped with *E. amarillans*. These two isolates were apparently relegated to a strictly asexual life cycle with only vertical transmission, yet semiartificial mating experiments (in which spermatia were raised in culture) indicated interfertility with their most closely related sexual species (A. Leuchtmann, C.L.S., and C.D.M., unpublished data). Thus, stroma expression most often requires the appropriate *Epichloë* species-by-host tribe interaction, and occasional jumps into new host species appears to render an isolate nonstromal and therefore effectively asexual.

4. ASEXUAL LINEAGES AND ASEXUAL SPECIES

4.1. Described Species

To date, 14 asexual *Neotyphodium* spp. have been formally described and named, of which 13 appear to be derived from *Epichloë* s. s. [The exception is *N. chilense*, for which the original classification as *Acremonium chilense* (Morgan-Jones et al., 1990) better fits morphological and phylogenetic information (G. A. Kuldau, A. E. Glenn, A. Leuchtmann, and C.L.S., unpublished data.) The first to be described were *N. typhinum* (as the anamorph of *E. typhina*), *N. coenophialum* from tall fescue (*Lolium arundinaceum* = *Festuca arundinacea*) (Morgan-Jones and Gams, 1982), and *N. lolii* from perennial ryegrass (*Lolium perenne*) (Latch et al., 1984). The latter two species have captured the most research interest because of their role in biological protection of common forage grasses and in toxicoses suffered by livestock that graze those grasses (Schardl and Phillips, 1997). Most other described *Neotyphodium* species likewise tend to be associated with individual grass species or species complexes: *N. uncinatum* and *N. siegelii* from *Lolium pratense* (= *Festuca pratensis*) (Craven et al., 2001a; Gams et al., 1990), *N. huerfanum* from *Festuca arizonica* (White et al., 1987), *N. chisorum*

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Position of midpoint root, when only *Epichloë* spp are included, is indicated by arrow 2. This phylogram was generated by neighbor-joining based on a Kimura 2-parameter distance matrix assuming ts/tv = 2.0.
from *Stipa eminens* (White and Morgan-Jones, 1987b), and *N. occultans* from species of the annual ryegrass complex, namely, *Lolium multiflorum, L. canariense, L. persicum, L. remotum, L. rigidum* var. *rigidum, L. rigidum* var. *rotboeliioides, L. subulatum, L. temulentum* (Moon et al., 2000). The *N. starrii* type is associated with *Festuca subulata*, but isolates from *F. arizonica* and *Bromus anomalus* were considered on a morphological basis to be the same species (White and Morgan-Jones, 1987c). Recently, several *Neotyphodium* species have been described from grasses indigenous to the Southern Hemisphere. First, *N. tembladerae* was described based on the type specimen from *Poa huecu*, with isolates from *Festuca argentina* and *F. hieronymi* considered to be the same species based on both morphological and molecular phylogenetic relationships (Cabral et al., 1999). Other Southern Hemisphere species are *Neotyphodium melicicola* from African *Melica decumbens*, and *N. australiense* and *N. aotearoae* from Australasian *Echinopogon ovatus* (Moon et al., 2002). A number of undescribed taxa considered to represent distinct species have yet to be formally named. In particular, results of an extensive study by Christensen et al. (1993) indicate that additional endophyte species are present in tall fescue and perennial ryegrass. The newly identified, but as yet undescribed, species in tall fescue have been designated FaTG-2 (“*F. arundinacea* endophyte taxonomic grouping 2”) and FaTG-3, and the additional species from *L. perenne* was designated LpTG-2.

In this chapter we discuss both described asexual species and those identified but not yet formally named. For the latter, we will use the convention of Christensen et al. (1993) by an abbreviation that refers to the host, followed by TG and a number to distinguish multiple species from a single host species (Table 1).

### 4.2. Nonhybrids

The simplest scenario for the evolution of asexual endophytes is a loss of the capability to complete the sexual life cycle. When *Epichloë* endophytes lose their ability to express stromata, on which both male and female structures develop (White et al., 1991), they necessarily lose sexual expression. There are, of course, alternative means by which the fungus may become asexual. For example, stromata may be formed but incapable of completing sexual development. Such a situation is easy to imagine, since many of these endophytes can disseminate clonally in host seeds and vegetative propagules and could thereby give rise to isolated clonal populations. The resulting bottleneck might cause a loss of one of the two mating types, thus precluding further sexual expression in such populations. Eventually, genes with roles in stroma formation or any subsequent sexual development may be lost. Loss of stroma formation should be selected based on the consequent increased production of seeds, the vehicle by which both
### Table 1 Hybrid Endophytes and Their Closest Nonhybrid Genome Contributors.

<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Closest nonhybrid relatives (clades)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eurasia/Northern Africa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FaTG-1</td>
<td>Festuca altissima</td>
<td>E. bromicola (e), E. typhina (k)</td>
</tr>
<tr>
<td>HboTG-2</td>
<td>Hordeum bogdani</td>
<td>E. bromicola (e), E. typhina (k)</td>
</tr>
<tr>
<td>HbrTG-2</td>
<td>Hordeum brevisubulatum</td>
<td>E. bromicola (e), E. typhina (k)</td>
</tr>
<tr>
<td>HeuTG-2</td>
<td>Hordelymus europaeus</td>
<td>E. bromicola (e), E. typhina (k)</td>
</tr>
<tr>
<td>N. coenophialum</td>
<td>Lolium arundinaceum</td>
<td>E. festucae (b), E. typhina (k), E. baconii (n)</td>
</tr>
<tr>
<td>FaTG-2</td>
<td>L. arundinaceum</td>
<td>E. festucae (b), E. baconii (n)</td>
</tr>
<tr>
<td>FaTG-3</td>
<td>L. arundinaceum</td>
<td>E. typhina (k), E. baconii (n)</td>
</tr>
<tr>
<td>LpTG-2</td>
<td>L. perenne</td>
<td>N. lolii (or E. festucae) (b), E. typhina (k)</td>
</tr>
<tr>
<td>N. uncinatum</td>
<td>Lolium pratense</td>
<td>E. bromicola (e), E. typhina (k)</td>
</tr>
<tr>
<td>N. siegelii</td>
<td>L. pratense</td>
<td>E. festucae (b), E. bromicola (e)</td>
</tr>
<tr>
<td>N. occultans</td>
<td>Lolium multiflorum, etc.</td>
<td>E. bromicola (e), E. baconii (n)</td>
</tr>
<tr>
<td>MciTG-1</td>
<td>Melica ciliata</td>
<td>N. “inebrians” (h), E. typhina (k)</td>
</tr>
<tr>
<td>North America</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. tembladoreae</td>
<td>Festuca arizonica</td>
<td>E. festucae (b), E. typhina (k)</td>
</tr>
<tr>
<td>FpaTG-1</td>
<td>Festuca paradoxa</td>
<td>E. amarillans (a), E. typhina (k)</td>
</tr>
<tr>
<td>PauTG-1</td>
<td>Poa autumnalis</td>
<td>E. elymi (f), E. typhina (k)</td>
</tr>
<tr>
<td>N. chisosum</td>
<td>Stipa eminens</td>
<td>E. amarillans (a), E. typhina (k), E. bromicola (e)</td>
</tr>
<tr>
<td>SroTG-1</td>
<td>Stipa robusta</td>
<td>E. festucae (b), E. elymi (f)</td>
</tr>
<tr>
<td>South America</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. tembladoreae</td>
<td>Poa huecu</td>
<td>E. festucae (b), E. typhina (k)</td>
</tr>
<tr>
<td>Australia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. australiens</td>
<td>Echinopogon ovatus</td>
<td>E. festucae (b), E. typhina (k)</td>
</tr>
<tr>
<td>Southern Africa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. meliciola</td>
<td>Melica decbens</td>
<td>E. festucae (b), N. aotearoae (j)</td>
</tr>
</tbody>
</table>

\(^a\) Clades are indicated in Figs. 4 and 5. Names and clades in **bold italic** indicate tub2 and/or tef1 sequences with a basal relationship to the extant sexual species.
grass and the asexual endophyte are disseminated. In certain North American Poa spp., stroma formation occurs fairly rarely (Leuchtmann and Clay, 1990), and those stromata have never been observed to develop sexual fruiting bodies (K. Clay, personal communication). However, most other asexual endophytes have altogether lost the stromal state.

A second means by which the stromal state can be lost is actually better supported by the data, and illustrates the point that the endophytes exist not as individuals but as components of symbiotic systems (symbiota). If, as seems common, an *Epichloë* species is closely adapted to a particular host species, genus, or tribe (Schardl et al., 1997), then individuals within that species might occasionally colonize hosts to which they have not fully adapted. In such novel symbiota the endophyte might exhibit developmental abnormalities such as an inability to form stromata (Schardl et al., 1991). Were it not for their vertical transmissibility they might rapidly go extinct, but with vertical transmission they might continue to survive and propagate indefinitely, albeit asexually. Hence, asexual endophytes might be *Epichloë* individuals that have been essentially trapped by a new host. Several likely examples of this are now apparent.

4.2.1. *Neotyphodium lolii*

Gene sequence data indicate that the common *L. perenne* endophyte, *N. lolii*, is derived from *E. festucae* (Schardl et al., 1994), and electrophoretic karyotyping has confirmed that *N. lolii* is a likely haploid (Kuldau et al., 1999). *Neotyphodium lolii* is a common symbiont of perennial ryegrass, with natural populations of the host exhibiting anywhere from 0 to 100% infection frequency (Lewis et al., 1997). Stromata have never been reported for *N. lolii*, and the only instances of stroma formation on *L. perenne* have been attributable to *E. typhina*. Given the extensive observations of perennial ryegrass in its native habitat in Europe, and where it has been transplanted and naturalized in North America, Australia, and New Zealand, it seems very unlikely that stroma formation would simply have gone unnoticed if it occurs. Furthermore, in repeated test matings to both mating types of *E. festucae*, perithecia (the sexual fruiting structures) have never developed (Schardl, unpublished data). Therefore, *N. lolii* is a clear example of an *Epichloë* lineage having lost the sexual state.

A fascinating recent finding is *E. festucae* symbiotic with *L. perenne* (Moon et al., 2000). This endophyte, like *N. lolii*, is not known to produce stromata, but contrasts with *N. lolii* in that it sporulates abundantly in culture, and the resulting conidia can act as spermatia (with mat-1 activity) when transferred to *E. festucae* stromata. These artificial matings result in production of mature fruiting structures (perithecia) with discernible ascospores, but the spores appeared nonviable. Nevertheless, based on this production of a morphologically discernible *E. festucae* state, we have classified the endophyte as such. Its occurrence in perennial ryegrass lends credence to a scenario whereby the grass was colonized
by *E. festucae*, which then evolved into *N. lolii*. Indeed, it is conceivable that the *E. festucae* identified in *L. perenne* and *N. lolii* both derived from the same original colonization, though this might well represent a separate and more recent infection by *E. festucae*.

Perennial ryegrass is host to two additional endophytes, *E. typhina* mentioned earlier, and a likely *N. lolii × E. typhina* (or *E. festucae × E. typhina*) hybrid (LpTG-2), discussed later.

4.2.2. *Neotyphodium typhinum* var. *canariense*

The only strain of *E. typhina* so far observed to be seed transmitted is that associated with *Poa nemoralis*. Strains from this host tend to have nearly identical DNA sequences, yet both mating types are represented in the population. This situation suggests that the *Poa nemoralis*-associated population may be highly inbred. In some *P. nemoralis–E. typhina* interactions essentially all inflorescences are choked and no seeds are produced. In others, however, normal seeds develop and the fungus is transmitted in those seeds (Chapter 6, this volume). Although sexual forms in this *E. typhina* subclade are found only in this host, there appear to be closely related asexual endophytes in other hosts. One is a rare endophyte in the annual ryegrass, *Lolium canariense*, which has therefore been described as *N. typhinum* var. *canariense* (Moon et al., 2000). Sequences of *tub2* introns and rDNA ITS regions indicate its close relationship to *E. typhina* from *P. nemoralis*, and there is no indication of hybrid origin. The extremely slow growth and lack of sporulation of *N. typhinum* var. *canariense*, and the fact that no stromata of this endophyte have been observed, indicate that it is truly asexual.

4.2.3. Nonstromal *Epichloë* Strains

Although the sexual stage of *E. bromicola* is known only on *Bromus erectus*, isolates from other host species have proven to be interfertile with *E. bromicola* if their conidia (raised in culture) are used as spermatia. In two instances these nonstromal forms were found in other species of *Bromus* (Leuchtmann and Schardl, 1998). In the third instance the endophyte was identified in *Hordelymus europaeus* (Chapter 6, this volume). Thus, although stromal *E. bromicola* appears restricted to the one host species in tribe Bromeae, the nonstromal associations are with other grass species in tribes Bromeae and Triticeae. Several other cases have been found of endophytes genetically very similar to known *Epichloë* spp., but not to the species that normally occur in those hosts. The occurrence of *E. festucae* in *L. perenne* was mentioned above. As another example, an endophyte derived from *E. amarillans* was found in *Elymus virginicus*, a common host of *E. elymi*. On the other hand, *E. elymi* appears to have infected *Bromus purgans* (tribe Bromeae) to give a nonstromal endophyte, whereas *E. elymi* stromata have been observed only on members of tribe Triticeae.
Thus, in every case, stromata develop only on certain hosts and not on these apparently new hosts, where the endophytes must propagate solely by vertical seed transmission. These observations lend credence to the concept of a “trapped endophyte,” whereby *Epichloë* spp. may sometimes infect hosts to which they are not closely adapted, such that they establish stable symbioses but are incapable of producing stromata on their new hosts (Schardl et al., 1991).

4.3. Hybrids

4.3.1. Identifying Hybrids: The Case of *Neotyphodium lolii* × *Epichloë typhina*

Most of the asexual endophytes tend to have two copies of most genes, with the copies derived from two different *Epichloë* spp. One of the most clear-cut and well-studied examples is the perennial ryegrass endophyte known as *Neotyphodium* sp. LpTG-2. Analysis of five genes by sequence and nine by isozymes has revealed that all but one, the rDNA, exist in two forms with relationships to two known endophytes, *N. lolii* and *E. typhina* (Collett et al., 1995; Schardl et al., 1994) (Fig. 3). In fact, the closest relative of the *E. typhina* contribution are isolates from perennial ryegrass, the same host as harbors *N. lolii* and LpTG-2. The genome size and electrophoretic karyotype of LpTG-2 is consistent with its derivation from a *N. lolii* × *E. typhina* hybridization (Kuldau et al., 1999; Murray et al., 1992). Furthermore, although the rDNA sequence is similar to that of *E. typhina*, the mitochondrial DNA profile appears similar to that of *N. lolii* (Schardl et al., 1994). It is common in direct analysis of the rDNA internal transcribed spacers to identify only a single sequence even in hybrids, probably because of the tendency of tandem rDNA copies to undergo gene conversion and thereby evolve concertedly (Ganley and Scott, 1998). The close relationship of LpTG-2 to extant representatives of its ancestral lineages, and the fact that LpTG-2 appears to be a relatively rare endophyte identified in only a single host population, suggest that it arose in a very recent hybridization event.

4.3.2. Identifying Hybrids: The Case of *N. uncinatum*

Not all genes from an original hybrid are necessarily retained in the extant endophyte. Since they are redundant, loss of the extra copies need not have a negative effect on the endophyte (unless important fitness-enhancing traits are also lost due to their presence on the same chromosome or genomic segment). An excellent example is *N. uncinatum*. In isozyme studies, only one of the eight enzymes investigated gave a multiband pattern in *N. uncinatum*, whereas other asexual endophytes tended to have multiband phenotypes for several enzymes (Leuchtmann and Clay, 1990). However, microsatellite analysis showed multiple alleles at two of the five loci analyzed (Moon et al., 1999). Sequence analysis of
Figure 3  Phylogeny of tub2 genes of Epichloë and Neotyphodium spp. showing phylogenetic position of the two gene copies in Neotyphodium sp. LpTG-2. Phylogram was generated using neighbor-joining based on a Kimura 2-parameter distance matrix assuming ts/tv = 2.0. Tree is midpoint rooted (left edge). Sequence clades are labeled as in Fig. 2. Hosts of E. typhina are abbreviated as follows: Lp = Lolium perenne (which is also host of LpTG-2 and N. lolii), Php = Phleum pretense, Ps = Poa silvicola, Ao = Anthoxanthum odoratum, Dg = Dactylis glomerata, Bp = Brachypodium pinnatum, Pn = Poa nemoralis and Pp = Poa pretense.
tub2 revealed only a single copy, and there was only a single copy detected for 

_\textit{tef1}_, but phylogenetic analysis placed these genes close to different _\textit{Epichloë}_ 

spp.: The tub2 gene was related to that of _\textit{E. typhina}_ from _\textit{P. nemoralis}_, whereas 

_\textit{tef1}_ grouped in the _\textit{E. bromicola}_ clade (Craven et al., 2001a). Furthermore, _\textit{act1}_ 

sequence analysis identified two copies, one phylogenetically close to the same _\textit{E. typhina}_ genotype, and another within _\textit{E. bromicola}_ (Craven et al., 2001a). 

Despite the tendency for _\textit{N. uncinatum}_ to have one copy of most genes, the 

conflict between its tub2 and _\textit{tef1}_ relationships, and the two _\textit{act1}_ copies, indicate 

that _\textit{N. uncinatum}_ is a hybrid.

Interestingly, analysis of rDNA also points to the same two ancestors for 

_\textit{N. uncinatum}_. Direct sequence analysis of rDNA ITS1-2 places it with 

_\textit{E. bromicola}_, but the sequence of a cloned PCR product grouped with _\textit{E. typhina}_ 

(C.L.S., unpublished data). Thus, even though _\textit{N. uncinatum}_ and other endo-

phytes show only a single dominant rDNA sequence in direct sequence analysis, 

this result illustrates that relics of hybrid ancestors may still be present in the 

rDNA. This is because rDNA exists in fungi (as in most eukaryotes) as tandemly 

repeated copies (Ganley and Scott, 1998). In _\textit{Fusarium}_ spp., this situation 

has permitted the maintenance of paralogs that occasionally switch relative 

dominance over the course of evolution (O’Donnell and Cigelnik, 1997).

To summarize, in _\textit{N. uncinatum}_ the two forms of _\textit{act1}_ and rDNA ITS 

regions, plus the conflict between tub2 and _\textit{tef1}_ phylogenies for this endophyte, 

strongly support a hybrid origin from _\textit{E. typhina}_ and _\textit{E. bromicola}_.

4.3.3. Other Simple and Complex Hybrids

Many hybrid _\textit{Neotyphodium}_ spp. were identified and characterized by analyses 

similar to those used for _\textit{N. uncinatum}_ and LpTG-2 (Table 1; Figs. 4 and 5). Of 

particular interest are the tall fescue endophytes—namely, _\textit{N. coenophialum}, 

FaTG-2 and FaTG-3 (Tsai et al., 1994)—plus _\textit{N. occultans}_ from the annual 

_\textit{Lolium}_ spp. (Moon et al., 2000). For each of these endophytes one ancestor is 

derived from clade _\textit{n},_ most closely related to _\textit{E. baconii}_ (Figs. 4 and 5). Various

\textbf{FIGURE 4} _\textit{Epichloë} and _\textit{Neotyphodium}_ tub2 gene phylogeny generated 

using neighbor-joining based on a Kimura 2-parameter distance matrix 

assuming ts/tv = 2.0. Tree is midpoint rooted (left edge). Clades are labeled 

as in \textbf{Fig. 2}, and an additional clade, _\textit{n}, is defined. Taxa are as in Table 1 with 

additional abbreviations as follows: _\textit{N. sp.} BpuTG-1 from _\textit{Bromus purgans}, 

_\textit{N. sp.} EhyTG-1 from _\textit{Elymus hystrix}, _\textit{N. sp.} EviTG-1 from _\textit{El. virginicus}, and 

_\textit{N. sp.} HeuTG-1 from _\textit{Hordeum europaeus}. Apparent hybrid taxa typically 

contained multiple gene copies (the exception being _\textit{N. uncinatum}_), and gene 

copy numbers are given in brackets.

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additional ancestors have contributed to the different species, with the hybrid formulas as follows: \( N. occultans = \text{clade } n \times E. bromicola \), FaTG-2 = clade \( n \times E. festucae \), FaTG-3 = clade \( n \times E. typhina \), and the complex hybrid \( N. coenophialum = \text{clade } n \times E. festucae \times E. typhina \).

Two endophytes have been identified with three genomes, or remnants thereof, a much rarer occurrence than the two-genome hybrids. One of the complex hybrids is \( N. coenophialum \) from tall fescue; the other is \( N. chisosum \) from \( S. eminens \). \emph{Neotyphodium coenophialum} has three \( tub2 \) copies and two \( tef1 \) copies (Figs. 4 and 5), and many of its isozyme and microsatellite loci exhibit multiple alleles (Leuchtmann and Clay, 1990; Moon et al., 2000). The genome size of \( N. coenophialum \), approx. 57 Mb, is almost twice the genome size of the sexual species, \( E. typhina \) and \( E. festucae \) (Kulda et al., 1999). Apparently much of the genetic redundancy generated by hybridization has been retained, but some has been lost. The genome of \( N. chisosum \) is less studied, but the three copies each of \( tub2 \) and \( tef1 \) (Figs. 4 and 5) support its origin by two hybridizations.

The occurrence of such complex hybrids as \( N. coenophialum \) and \( N. chisosum \) suggests that endophyte hybridization occurs via somatic fusion followed by karyogamy (fusion of nuclei). The alternative, sexual hybridization, would be difficult to explain because it would require a heteroploid but sexual species undergoing further hybridization. Although hybridization might conceivably have occurred in the pedigrees of some sexual strains (Schardl et al., 1997), aneuploidy in sexual \emph{Epichloë} species is rare and limited, in that very few duplicated genes are in evidence (Leuchtmann and Clay, 1990). However, an extensively heteroploid but sexual ancestor would have to be invoked to hypothesize a sexual hybridization that gave rise to \( N. coenophialum \) or \( N. chisosum \). Since this is unlikely, somatic hybridization is indicated for these two species, and is probably the usual mechanism for all hybrid \emph{Neotyphodium} species.

4.3.4. Predominance of Hybrids

A broad survey was conducted of asexual endophytes worldwide (Figs. 4 and 5). The criterion for identifying these as asexual was that they failed to produce

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure5}
\caption{\emph{Epichloë} and \emph{Neotyphodium} \( tef1 \) gene phylogeny generated using neighbor-joining based on a Kimura 2-parameter distance matrix assuming \( ts/tv = 2.0 \). Tree is midpoint rooted (left edge). Clades and taxa are labeled as in Fig. 4. Apparent hybrid taxa typically contained multiple \( tef1 \) copies (exceptions being \emph{N. uncinatum}, \emph{N. melicicola}, \emph{N. sp. HBO-TG-1}, \emph{N. sp. HbrTG-1}, \emph{N. sp. HeuTG-2}, and \emph{N. sp. LpTG-2}), and gene copy numbers are given in brackets.}
\end{figure}
stromata on host plants. A total of 28 such asexual species had clearly distinct evolutionary origins; of these, 19 had the hallmarks of interspecific hybrids (Table 1). Hybrids were identified in both hemispheres, and on all major continents (except Antarctica, which was not sampled). Furthermore, hybrids were identified from grass hosts in five tribes of the subfamily Pooideae, including the more deeply rooted tribes Stipeae (*Stipa* spp.) and Meliceae (*Melica* spp.), sister tribes Poeae (*Festuca, Lolium* and *Poa* spp.) and Aveneae (*Echinopogon*), and the Triticeae (*Hordeum* spp. and *Hordelymus europaeus*).

### 4.4. Patterns in Hybrid Relationships

#### 4.4.1. Relationships with Extant Sexual Species

Of considerable interest is the tendency for gene sequences from hybrids often to lie outside and basal to the clades of extant sexual species. The most clear-cut examples in the *tub2* phylogeny (Fig. 4) are *E. amarillans*-related *tub2* sequences from *N. chisum* and an endophyte of *Festuca paradoxa*; *E. bromicola*-related sequences from *N. chisum* and endophytes of *F. altissima*, *Hs. europaeus*, *H. bogdanii*, and *H. brevisubulatum*; and *E. baconii*-related sequences from *N. occultans*, *N. coenophialum*, FaTG-2 and FaTG-3. In addition, *tef1* analysis suggests deep rooting of *E. elymi*-related genomes in *Poa autumnalis* and *Stipa robusta* endophytes, and of *E. festucae*-related genomes in *N. australiense*, *N. siegeli* and FaTG-2 (Fig. 5). In contrast, most nonhybrid asexual strains were more closely related to extant sexual species. In several instances, isolates from the sexual species formed groups that were paraphyletic to nonhybrid asexual isolates; i.e., each nonhybrid asexual isolate was contained within a clade or subclade that encompassed its most closely related sexual species. Thus, the nonhybrid from *Hs. europaeus* (HeuTG-1) appeared to be much more recently derived from *E. bromicola* than were the *E. bromicola*-related genomes of HeuTG-2, *N. occultans*, *N. chisum*, FaTG-1, HboTG-1, and HbrTG-1 (Fig. 4). Similar contrasts were especially evident in the *tef1* phylogeny (Fig. 5): *tef1* in the nonhybrid EviTG-1 versus the clade *a* copies from *N. chisum* and FpaTG-1; and *N. lolii* *tef1* versus clade *b* copies in *N. siegeli*, *N. australiense* and FaTG-2. In fact, wherever sequences from sexual and asexual species occurred together in a clade, the more deeply rooted sequences were those of hybrids, with the sole exception of BpuTG-1 (from *Bromus purgans*). Conversely, most hybrids had at least one gene copy relatively deeply rooted in its respective clade (highlighted in Table 1).

The origins of hybrids and nonhybrids in clades *h* and *j* remain unknown because there is no sexual species associated with these clades. However, the relationships of nonhybrids *N. aotearoae* and *N. “inebrians”* with hybrid endophytes in Eurasian and South African *Melica* spp. suggest that a sexual species for each of these clades either has not yet been sampled or has become
extinct. Interestingly, both nonhybrids are extremely slow-growing and nonsporulating in culture, whereas the hybrid endophytes in *Melica* spp. grow and sporulate in culture much as do sexual *Epichloë* spp. (Moon et al., 2002). These observations suggest that hybrids may tend to be more vigorous than the nonhybrid asexual endophytes.

### 4.4.2. Phylogeography

Patterns of phylogeography differ somewhat between nonhybrids and hybrids. Although asexual species are distributed worldwide, sexual species have so far been identified only in the Northern Hemisphere. Furthermore, each sexual species has so far been associated with either Eurasia or North America, but not both (except where probably transported by humans). Nevertheless, the overall phylogeny fails to indicate any obvious region of origin or any region-specific clades except for the Eurasian *E. typhina* complex. Interestingly, the Eurasian species *E. baconii* and *E. festucae* group with the North American *E. amarillans*, and the North American *E. elymi* and *E. glyceriae* group with Eurasian *E. bromicola* (Fig. 2) (Schardl et al., 1997). Thus, in contrast to the situation with the *Gibberella fujikuroi* complex (O’Donnell et al., 1998), there is no indication of cladogenesis associated with vicarious events such as breakup of Pangea and separation of the continents. Nevertheless, geographic isolation as well as host specialization is probably important in speciation. This is illustrated by the intersterility between *E. baconii* and *E. amarillans*, phylogenetically related species that occur on closely related grasses but on different continents. However, the contributions of *Epichloë* spp. to hybrid endophytes paint a picture of considerable communication of species around the poles and between hemispheres.

There is a strong but not absolute tendency for hybrids in Europe to have ancestors most closely related to European species, but North American and other hybrids often have contributions both from North American species and from species now common in Europe. In fact, Eurasia-associated spp. *E. typhina* and *E. festucae* show up as likely contributors to hybrids throughout the Northern and Southern Hemispheres. These relationships suggest that *E. typhina*, *E. festucae* and perhaps other *Epichloë* spp. may have been very widespread previously, but later became rare or extinct outside Eurasia.

Although no sexual *Epichloë* species have yet been found in the Southern Hemisphere, the association of *N. aotearoae* with that region, both as a nonhybrid in Australasia and as a hybrid component in southern Africa, suggests that a closely related *Epichloë* species may be—or may have recently been—indigenous to the southern continents. This observation also indicates considerable migration of the ancestors of genomes that now constitute both hybrid and nonhybrid *Epichloë/Neotyphodium* species.
5. EVOLUTIONARY PROCESSES

5.1. Species Concepts

5.1.1. Morphospecies

Traditionally species have been regarded simply as life forms with distinguishable forms (i.e., morphologies). Such a definition is obviously very broad and ambiguous, but the basis for refining the definition has been a matter of contention at the very core of biology. The formal process of describing morphological species is rooted in a pre-Darwinian concept of hierarchical relationships among living forms, sometimes leaving little room for the incorporation of new science. In fact, fungal taxonomy represents an extreme at which the organism itself is not actually named, only its developmental states. The teleomorph is the form taken by the sexual state, the anamorph by the asexual state. Generally a fungus has only one teleomorph, and the teleomorph is often the more complex (but rarer) form exhibited. Multiple anamorphs may be exhibited by an individual. It seems reasonable, then, that the teleomorph name, when available, is ascribed to whole organism as the “holomorph” (Greuter et al., 2000). Hence, the 10 *Epichloë* species s.s. that have been described are regarded as holomorphic species, but asexual relatives are separately classified in the form genus *Neotyphodium*. On the face of it, this arrangement says little about the evolutionary relationships between these genera and species therein.

5.1.2. Biological Species

The concept of a species as an inclusive gene pool has a certain attraction among fungal biologists who deal with a paucity of morphological features on which to base species distinctions. Mayr (1974) formalized the concept of a biological species to include all potentially interbreeding members. For fungi, one may extend this definition to include both sexual and parasexual interbreeding; the distinguishing feature of parasexual gene exchange being that meiosis is not involved (Caten, 1981). However, difficulties in application to many fungi for which sexual or parasexual exchange may be difficult to document have largely caused biological species concepts to go out of favor among mycologists (as, indeed, the concept has become less favored for other groups of organisms). One of the most obvious problems is posed by strictly clonal organisms, of which there are many in the microbial world and some even among large animals and plants. Clonal organisms simply have no place in a strict biological species concept. Nevertheless, it is desirable for any modern species concept to take into account biological species, which has a basis in a very real and common phenomenon, barriers to genetic exchange between populations.
5.1.3. Phylogenetic Species

The essence of the phylogenetic species concept is that species be circumscribed on an evolutionary basis (Cracraft, 1997). Identification of phylogenetic species employs sets of shared traits. The traits chosen must distinguish species by having the appropriate level of polymorphism, and should reliably reflect evolution. It is not necessary that all such traits be discerned from direct analysis of the genetic material (DNA) or gene products (e.g., proteins), but these macromolecules represent very rich sources of polymorphic characters, and a sophisticated set of computer tools has been generated to utilize DNA and protein sequences for phylogenetic inferences (Swofford et al., 1996).

A strength of the phylogenetic species concept—namely its broad applicability—is also a weakness because, like the morphospecies concept, there is considerable room for judgment in deciding what constitutes the same or different species. In fact, there are actually several proposed phylogenetic species concepts (Mayden, 1997). Most require that species be monophyletic units, a criterion that has been criticized as overly restrictive and not necessarily reflective of biological realities (Avise and Wollenberg, 1997). In analysis of *Epichloë* species, many of the biological species appeared as good phylogenetic species and were monophyletic; however, there were difficulties in the application of either concept when considering the three species of the *E. typhina* complex: *E. typhina*, *E. sylvatica*, and *E. clarkii* (Craven et al., 2001b; Leuchtmann and Schardl, 1998; Chapter 6, this volume). Reconciliation of these conflicts with the phylogenetic species concept can be done either by including the whole *E. typhina* complex in one species, or by abandoning any requirement of monophyly and arranging species as best fits both their interfertility relationships and evolution.

The phylogenetic species concept provides an excellent practical solution for circumscribing morphologically cryptic asexual species, such as the many nonstromal *Neotyphodium* species. Again, sequences of DNA (or RNA or protein) provide a rich source of phylogenetically informative data. However, application of the phylogenetic species concept for most of these asexual endophytes poses a new problem. Specifically, the majority of *Neotyphodium* spp. have apparent hybrid origins, with two or more *Epichloë* spp. in their pedigrees. As illustrated above, this is generally evident by the presence of multiple gene copies and the sequence relationships of the copies to each other and counterparts in sexual *Epichloë* spp. The phylogenetic species concept appears applicable if each particular set of multiple gene copies is considered an evolutionarily based character state shared with members of the same species. Thus, for example, *N. tembladerae* can be characterized in part by the presence of two *tub2* and two *tefl* genes, and their close relationships to certain genotypes of *E. typhina* and *E. festucae* (Table 1; Figs. 4 and 5). Whereas other
E. typhina × E. festucae hybrids are evident (Figs. 4 and 5), sequence polymorphisms indicate that different genotypes of these ancestral species contributed to these other hybrids, justifying their classification as distinct species. An example is the distinction of N. australiense from N. tembladerae. In contrast, the genotype of a recently discovered F. arizonica endophyte is so far consistent with its inclusion in a phyllogenetically based N. tembladerae (Table 1).

We therefore advocate use of a phylogenetic species concept, but without the constraint of monophyly, to provide an inclusive framework for identifying species of sexual and asexual endophytes. The great advantage of this concept is that it requires an evolutionary basis, and thereby requires an elucidation of the very interesting and varied patterns of speciation among these symbionts.

5.2. Possible Speciation Mechanisms for Endophytes

5.2.1. Co-Cladogenesis and Host-Driven Speciation

Current information strongly implicates host specialization as an important driving force for speciation of the sexual endophytes. With the exception of the E. typhina complex and a Koeleria-associated E. festucae, each phylogenetic species is associated with a single host tribe and closely related species or genera within that tribe (Chapter 6, this volume). Even within the E. typhina complex, gene trees resolve clades associated with certain host species and genera, such as Brachypodium spp., Holcus lanatus, Poa nemoralis, and Poa pratensis. Intriguingly, the population associated with Brachypodium sylvaticum has extremely low interfertility even with the closely related E. typhina strains on Bp. pinnatum, and has therefore been described as a separate species, E. sylvatica. Furthermore, some isolates of E. typhina appear adapted to certain hosts and incompatible with others (Chung et al., 1997), suggesting incipient speciation driven by host specialization. Among the possible mechanisms for fungal speciation, the following two scenarios can be envisaged for evolution of host-specific phylogenetic species.

First, specialization to new hosts (host shifts) might drive speciation. Evolution of a clonal lineage specialized to a new host is much simpler than establishment of a new host-adapted sexual population. In either case the process would begin with an ascospore that happens to land on a new host plant at the right time and place (e.g., onto the stigma just after exertion), and with a genotype compatible with the plant. If the association has the potential to transmit via seeds, the endophyte will have thereby established a new niche and will be clonally propagated in that niche. However, since the sexual states of Epichloë spp. are heterothallic (require different mating types to cross) (White and Bultman, 1987), further colonization by the second mating type is needed to establish a new host-specialized, sexual population. An observation that suggests how this may happen is that, in most cases both within and outside the E. typhina
complex, the genotypes associated with a host species are all very closely related, yet both mating types are present (Leuchtmann and Schardl, 1998). For those species or clades that have arisen by host shifts, this situation can best be explained by intensive inbreeding. Thus, genotypes that are compatible with a new host would tend to breed productively only with each other. How this would happen seems apparent: most matings between isolates that are specialized to different hosts would give progeny whose compatibility with either parental host is reduced compared to the parents. This was observed experimentally in genetic analysis of host-specialized E. typhina strains and their progeny (Chung et al., 1997). Thus, progeny of matings between isolates on the same host species stand a greater chance of survival provided they land on that host. Inbreeding would be essentially inevitable. This process operating in the E. typhina complex seems most likely to explain the emergence of E. clarkii, E. sylvatica and host-specific clades of E. typhina. The process may also be at work in other Epichloë clades, as suggested by the relationship of Festuca and Koeleria-associated E. festucae isolates (Craven et al., 2001b). Thus, colonization and inbreeding explains at least some of the extant Epichloë–grass relationships.

The second scenario involves Epichloë speciation coincident with host cladogenesis. This may occur simply by geographic isolation of host populations together with their symbiont populations. When they eventually merge again (geographic barriers are eliminated), both host and fungal populations may have diverged sufficiently to constitute pairs of sister species. It is also possible that host cladogenesis sometimes causes Epichloë speciation. As ancestral grass taxa give rise to daughter taxa, and these diverge over time, Epichloë lineages may be forced on diverging trajectories of adaptation to the sister host taxa, limiting gene flow between different host-adapted populations and eventually differentiating species. It seems apparent, in fact, that different endophytes must specialize to their different hosts, since even the mutualistic, asexual species tend to exhibit reduced or no compatibility when moved experimentally to new host species, even when the new hosts are close relatives of the original hosts (Koga et al., 1993). This scenario also fits well with relationships within the Epichloë main group, in which most species are adapted to different host tribes, and those that occur on the same tribe are on different continents (E. baconii in Eurasia, and E. amarillans in North America). Furthermore, the branching order of Epichloë main-group species in Fig. 2 closely reflects the branching order of their host tribes.

Thus, two contrasting modes of population differentiation are suggested as the prevailing processes in evolution of the E. typhina complex and the Epichloë main group. In the former, it appears that new genotypes (arising by sexual recombination) colonize new hosts, and subsequent inbreeding enhances adaptation for the new host. In the latter, the Epichloë main group, co-cladogenesis with hosts seems to have been common. In both cases, adaptation to host species
has entailed both the capability to maintain stable symbiosis and the ability to undergo sexual development (stroma production) on the new host. However, a very large number of endophyte species require no stroma production, being strictly seed transmitted. How do speciation and host adaptation of the asexual endophytes resemble or differ from these processes in sexual species? This is an interesting question with some surprising answers we now address.

5.2.2. Clonal Speciation

The simplest process by which asexual species may arise is by losing the capability or opportunity for sexual recombination. As is common among fungi and other eukaryotes, the sexual cycle is developmentally complex and often requires a specific set of environmental conditions. In the case of an *Epichloë* sp. that has adapted to one host, a new host may constitute an environment that is nonconducive to stroma production. *Epichloë* isolates that enter such nonstromal symbioses would be removed from the gene pool, since stromata are the source of both male and female components. [But note that epiphytic hyphae discussed by White et al. (1996) conceivably give rise to spermatia; however, whether these can be vectored by flies or otherwise and thereby participate in matings is unknown.] As pointed out earlier, several nonhybrid asexual lineages are probably trapped endophytes resulting from host shifts.

5.2.3. Interspecific Hybridization and Selection

Although asexual endophytes can obviously arise by loss of sexual expression, especially following host shifts, a majority of these endophytes have more complex derivations as interspecific hybrids (Table 1). Why are there so many interspecific hybrids among the *Neotyphodium* species? Does clonality promote or select for hybrids, or does hybridization cause the loss of sexual expression? To address these questions the order of events in evolution of the hybrids must be inferred. In one scenario, an asexual endophyte may arise directly from a haploid *Epichloë* genotype, perhaps as a trapped endophyte, then the descendants of that endophyte/host association may become coinfected with another *Epichloë* genotype, which may subsequently hybridize with the resident endophyte. If the hybrids have a particular selective advantage over the nonhybrid progenitors, the endophyte population in that host species will become dominated by one or more hybrids. An alternative scenario would hold that the most common route by which asexual endophytes are generated is via interspecific hybridization of two sexual strains. This alternative would not necessarily require a selective advantage of hybrids over nonhybrids, but would nevertheless also predict hybrids to dominate.

Some evidence suggests that interspecific hybridizations can indeed cause loss of sexual expression. In the host species, *L. perenne*, a hybrid (LpTG-2) was identified whose closest known *E. typhina* genotype readily produces sexual
stromata on the same grass species (Schardl et al., 1994). The obvious result of this hybridization was a seed-transmissible endophyte that lacks the stromal expression of its *E. typhina* ancestor. Although the other ancestor of LpTG-2 was very closely related to *E. festucae*, evidence suggests it was more likely an asexual derivative of *E. festucae*, namely, *N. lolii* (Schardl et al., 1994). Nevertheless, it is noteworthy that the hybrid endophyte produces no stromata on *L. perenne* and has proven incapable of mating with stromata of closely related *E. typhina* or *E. festucae* strains (with appropriate mating types). Thus, hybridization apparently eliminated sexual expression, raising the possibility that other asexual species arose by hybridization of two sexual species. However, a more likely scenario is that most hybrids arose by fusion of an asexual *Neotyphodium* sp. with an *Epichloë* sp. that has co-infected the plant, and that such hybrids have a selective advantage over their nonhybrid asexual progenitors.

### 5.3. Mechanisms of Selection for Hybrids

#### 5.3.1. Evidence for Enhanced Competitiveness of Hybrids

Two lines of evidence suggest strong selection of the hybrids over nonhybrid asexual endophytes. First, phylogenies tend to deeply root sequences from the hybrids compared to nonhybrids (note phylogenetic positions of hybrids and nonhybrids in Figs. 4 and 5, summarized in Table 1). In fact, all but a few nonhybrids appear to be recently derived from known *Epichloë* species, and the rarity of long-lived, nonhybrid asexual lineages suggests that they have a fitness disadvantage. Second, some grass species have multiple hybrid endophytes that share a common ancestor, but that ancestor has apparently gone extinct in those grasses. For example, two endophytes of *L. pratense* may well share an *E. bromicola* ancestor, but one of these endophytes (*N. siegelii*) is an *E. bromicola × E. festucae* hybrid, and the other (*N. uncinatum*) is an *E. bromicola × E. typhina* hybrid (Craven et al., 2001a). However, none of the progenitor nonhybrids—*E. bromicola*, *E. typhina*, or *E. festucae*—has been observed in extant *L. pratense* populations despite an intensive survey (Craven et al., 2001a). A more obvious case is presented by endophytes in *L. arundinaceum* (Table 1). These endophytes constitute three hybrid species, all with a genome contribution from clade *n*, a relative of *E. baconii* (Tsai et al., 1994). One of these endophytes (FaTG-2) is clade *n × E. festucae*, and a second (FaTG-3) is clade *n × E. typhina*. The third is the complex hybrid, *N. coenophialum*, consisting of clade *n* with *E. typhina* and *E. festucae* genotypes distinct from those in FaTG-2 and FaTG-3. These relationships suggest that a clade *n* endophyte persisted in an ancestor of *L. arundinaceum* long enough to be involved in several hybridizations, yet the original nonhybrid endophyte has nevertheless been supplanted by these hybrid derivatives.
Phylogenetic tracking of the clade \(n\) genome with host maternal lineages indicates that this genome is a relic of an ancient asexual endophyte. Clade \(n\) sequences exist in the three hybrid endophytes of \(L.\ arundinaceum\) as well as in \(N.\ occultans\), the hybrid endophyte of annual \(Lolium\) spp. (Moon et al., 2000). Phylogenetic tracking is indicated by comparative phylogenetics of the clade \(n\) component of these four hybrid endophytes with the maternally inherited chloroplast genome (cpDNA) (Fig. 6). Interestingly, cpDNAs among \(L.\ arundinaceum\) plants are very diverse, and some are closer to \(L.\ multiflorum\) than to other \(L.\ arundinaceum\) cpDNAs. These relationships are mirrored in the phylogenies of the clade \(n\) components of the endophytes. Plants with \(N.\ coenophialum\) pair with \(L.\ multiflorum\), reflecting the pairing of \(N.\ coenophialum\) with \(N.\ occultans\). Likewise, Mediterranean \(L.\ arundinaceum\) plants harboring FaTG-2 and FaTG-3 have related cpDNAs, reflecting the close relationships of clade \(n\) sequences in those two endophytes. These results indicate that the ancestral clade \(n\) genome existed in a common ancestor of four endophytes, and persisted in those endophyte lineages even during host speciation. Surprisingly, however, the clade \(n\) genome has so far never been found in a nonhybrid. Thus, not only has clade \(n\) undergone several hybridizations, the resulting hybrids have clearly predominated and any nonhybrid progenitor species has become rare or extinct.

As mentioned previously, the endophytes of \(L.\ pratense\) similarly include multiple hybrids (\(N.\ uncinatum\) and \(N.\ siegelii\)) but lack the progenitor nonhybrids. The situation is very different in \(L.\ perenne\), however, since the nonhybrid \(N.\ lolii\) predominates over the hybrid LpTG-2. Perhaps in this case the hybridization was a much more recent event, as suggested by the phylogenetic and genomic analysis (Kuldau et al., 1999; Schardl et al., 1994), and in future LpTG-2 (and/or other emerging hybrids) may become dominant in the \(L.\ perenne\) population.

The picture emerges, then, of asexual nonhybrids most frequently arising from \(Epichloë\) spp. by host jumps, then subjected to hybridization when the host plants are co-infected by other \(Epichloë\) genotypes. Furthermore, the resulting hybrids apparently supplant the ancestral nonhybrids on an evolutionarily rapid time scale. There are two obvious possibilities whereby the hybrids may enjoy a fitness advantage over nonhybrid clonal endophytes. First, hybridization may bring together various characteristics that evolved independently in the ancestral lineages, essentially pyramiding adaptive traits. Alkaloid biosynthetic capabilities in particular might be selected. Second, hybridization may overcome negative effects of accumulated deleterious mutations that could otherwise be removed by the sexual cycle.
5.3.2. Selection on Alkaloid Biosynthesis Capabilities

Evidence suggests that alkaloid biosynthesis capabilities enhance the fitness of many asexual endophytes, probably by enhancing deterrence against herbivores. *Epichloë/Neotyphodium* spp. produce four known classes of antiherbivore alkaloids: ergot alkaloids (ergovaline and simpler clavine and lysergyl alkaloids), indole diterpenes (lolitrems and related tremorgens), 1-aminopyrrolizidines (lolines), and a pyrrolopyrazine (peramine) (Porter, 1994). The number of different alkaloid classes detected from any one endophyte varies between zero and three (Siegel et al., 1990). Three alkaloids are produced by each of the following species: *N. coenophialum* produces lolines, ergot alkaloids, and

![Diagram](image-url)
peramine, whereas *N. lolii* (a nonhybrid) and certain *E. festucae* genotypes produce lolitremes, ergot alkaloids, and peramine (it is noted that certain other *E. festucae* genotypes produce the fourth class, namely, lolines; Leuchtmann et al., 2000). Thus, there is no obvious tendency for hybrids to have more biosynthetic capabilities than nonhybrids. However, although most asexual endophytes studied to date produce lolines, ergot alkaloids, or both (Blankenship et al., 2001; Craven et al., 2001a; Miles et al., 1996, 1998; Petroski et al., 1992; Siegel et al., 1990; TePaske et al., 1993), many sexual strains produce no alkaloids or only the least potent antiherbivore alkaloid, peramine (Leuchtmann et al., 2000).

Since ergot alkaloid biosynthesis genes have been cloned (Panaccione et al., in press; Chapter 13, this volume), and a genetic marker has been linked to loline biosynthesis (Wilkinson et al., 2000), it has become possible to survey a large collection of sexual and asexual endophytes for these genes and markers. Intriguingly, with very few exceptions, only known producers of these alkaloids have the corresponding genes and markers (Wang, 2000; Wang and Schardl, 2001; M. J. Spiering and C. L. S., unpublished data). Also of particular interest is that *E. festucae* is the only sexual species known to produce ergovaline, lolitremes, or lolines (Leuchtmann et al., 2000; Siegel et al., 1990). This and the large number of hybrids with *E. festucae* components raise the intriguing question of whether some hybrids are selected based on their capability to produce these potent antiherbivore alkaloids. If hybrids are indeed selected based on their unique metabolic capabilities, one might expect an association between alkaloids and these *E. festucae* genome components, but this expectation so far fails to conform to the data. For example, lolines are produced by the apparent nonhybrid *N. aotearoae*, which is not closely related to *E. festucae*. Likewise, *N. uncinatum*, *N. occultans*, and PauTG-1 produce lolines, but have no apparent *E. festucae* contribution to their hybrid pedigrees.

Curious distributions and relationships of *dmaW* genes, which encode the first determinant step in ergot alkaloid biosynthesis, may suggest how phylogenetic and metabolic relationships have come to be so disparate. Notably, the presence or absence of *dmaW* fails to track either *Epichloë* or *Neotyphodium* phylogenies. This gene was detected in only three distantly related sexual species: *E. festucae*, *E. glyceriae*, and *E. clarkii* (Wang, 2000). Either the gene was present in the ancestor of genus *Epichloë*, but was subsequently lost from most *Epichloë* lineages independently (not a very parsimonious explanation), or *dmaW* has been transferred horizontally between *Epichloë* species. Results from nonhybrid asexual species add further insight. Although the *N. lolii* *dmaW* may have been derived from its *E. festucae* ancestor, the *dnaW* gene in *N. "inebrians"* [which makes copious amounts of simple lysergyl derivatives (Miles et al., 1996)] is very distantly related to those of the *Epichloë* spp. (Wang, 2000). In fact, the relationship between these different *Neotyphodium dnaW* genes is as
great as between the homologs from three different genera: *Epichloë*, *Balansia*, and *Claviceps* (Fig. 7), strongly suggesting a history of horizontal gene transfer.

If horizontal transfer of metabolic genes occurs in the Clavicipitaceae, such transfers have left few, if any other indicators of hybridization. A likely explanation is that hybridizations in this group are even more widespread than so far indicated by housekeeping gene phylogenies, and that some hybrids revert largely to the genotype of one ancestor while retaining new alkaloid synthesis genes. Comparative genomic analysis of Clavicipitaceae would likely reveal whether this is true and why. Possibilities are that the genes are on mobile clusters flanked by transposons, that they are associated with chromosome ends (telomeres), or that they are on conditionally dispensible chromosomes (Kistler and Miao, 1992).

5.3.3. Muller’s Ratchet

In a landmark paper, Muller (1964) demonstrated that clonal lineages may be subject to reduce fitness due to a relentless increase in the load of deleterious mutations, and that sexual recombination would tend to reduce that load. The progressive increase in deleterious mutations has become known as Muller’s ratchet, and there is evidence for the ratchet in nonrecombining genomes present in eukaryotic cells, namely, mitochondrial DNA (Lynch, 1997; Moran, 1996), sex chromosomes, and *Drosophila* chromosomes that are prevented from recombining (Rice, 1994). A potential consequence of the ratchet is extinction of clonal lineages (Lynch et al., 1993). Some models indicate that clonal species
with very large effective population sizes will escape the ratchet (Bidochka and De Koning, 2001). However, clonal grass endophytes likely have very low effective population sizes, no greater than that of their hosts. In fact, although a large population of fungal genomes exists in any endophyte-infected plant, very few hyphal strands colonize each new tiller or seed and are propagated to the next host generation (Freeman, 1904; Philipson and Christey, 1986; Schmid et al., 1999; White et al., 1991). Such bottlenecks could greatly reduce the effective population size of endophyte genomes in a host population, and may enhance the effect of Muller’s ratchet. We note, however, that if the endophytes are strongly subject to selection at the bottleneck, the effect of Muller’s ratchet might actually be ameliorated (Bergstrom and Pritchard, 1998). In the absence of information about the nature of selection at this stage, and the reason why so few hyphae colonize host propagules, the relative contribution of Muller’s ratchet remains unknown.

If Muller’s ratchet provides a significant basis for selection on asexual endophytes, then interspecific hybridization should reduce the ratchet effect, and thereby result in the selective advantage of hybrid over nonhybrid clonal lineages. This is envisioned in part because multiple copies of genes are apparent in hybrid endophyte genomes. Thus, multiple deleterious mutations may be required to negatively affect functions of redundant genes, whereas in nonhybrid endophytes there is a higher probability for each mutation to have a negative affect (note, however, that even in diploids some single mutation events may have dominant negative effects). Furthermore, in the *Epichloë/Neotyphodium* system each hybridization infuses the asexual endophyte with a genome that has recently undergone meiotic recombination, improving its chance of being purged of multiple deleterious mutations. One may predict that, as redundant copies are subsequently lost (as seems to have happened extensively with *N. uncinatum*), those of the more recently infused genome are more likely to be retained. There are insufficient data at present to test this prediction.

Currently the evidence does not strongly favor either possibility for hybrid fitness: selection on pyramided traits such as alkaloids, or Muller’s ratchet. It seems likely that both of these forces may be operative separately in some circumstances, and together in others.

6. CONCLUSIONS

The genera *Epichloë* and *Neotyphodium* are extraordinary among symbiotic and parasitic microbes for a number of reasons. The biology and life histories of these endophytes make them excellent models to study relationships and trade-offs between host and symbiont fitness, since their symbioses with grasses span a continuum of relative antagonism and mutualism. In no other well-studied system is there such a close relationship among mutualists, pleiotropic symbionts, and
antagonists (likewise, a close relationship of the hosts). Furthermore, their well-defined life cycle and ecological niches have permitted scrupulous evaluation of many aspects of endophyte biology, including sexual expression, transmission modes, metabolic capabilities, host specialization and interactions, genome organization, biological species, and phylogenetic species. Clearly, all of these aspects have profound influence on evolutionary processes, as is dramatically illustrated by three different patterns of evolution associated with three different life history strategies: first, those that are only horizontally transmitted and obligately sexual often exhibit indistinct boundaries of biological and phylogenetic species relationships, sometimes associated with broad host range; second, those that have mixed vertical and horizontal transmission strategies, respectively associated with clonal and sexual reproduction modes, tend to track host phylogenies as expected for co-cladogenesis; and third, those that are locked into asexual propagation via vertical transmission are most often hybrids, apparently because this life history strategy disproportionately favors the hybrids over their nonhybrid progenitors. Other obligately symbiotic fungi exhibit interesting parallels to these endophytes, particularly in production of various secondary metabolites (Culberson et al., 1992, 1993; Lawrey et al., 1999) and unusual genome organizations (Pringle et al., 2000; Sanders et al., 1996; Vandenkoornhuyse and Leyval, 1998). Therefore, any further elucidation of the evolutionary interplay between these various characteristics and mutualism in the grass–endophyte systems is very likely also to shed light on evolution of other important symbioses.

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Interkingdom Host Shift in the *Cordyceps* Fungi

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1. **INTRODUCTION**

In this chapter we describe the phylogenetic reconstruction of drastic host shifts in the *Cordyceps*, a major genus of principally entomoparasitic fungi in the family Clavicipitaceae, and discuss the ecological and evolutionary implications of the findings. The results described in this chapter have already been published (Nikoh and Fukatsu, 2000), and the reader should refer to the original article for details.

2. **HOST SPECIFICITY VERSUS HOST SHIFT**

There are a number of taxa whose members are exclusively endoparasitic to other organisms: parasitoid wasps (Godfray, 1994; Hawkins, 1994), parasitic helminths (Despommier and Karapelou, 1987; Kearn, 1998), entomopathogenic
fungi (Samson and Evans, 1988; Tanada and Kaya, 1993; Shimizu, 1994), parasitic protozoans (Kreier, 1993; Coombs et al. 1998), rickettsial endoparasitic bacteria (O’Neill et al., 1997), and many others. In general, these endoparasites show more or less strict host specificity, in which an endoparasite can infect and utilize a particular range of host organisms, or sometimes only a particular species of host. The host specificity is likely to be the natural outcome of the endoparasitic life, in which endoparasites have to develop highly specific and sophisticated mechanisms to recognize the host, to enter the host body, to avoid the host’s immune system, to survive and proliferate under special endoparasitic environments, to synchronize their life cycle parameters to those of the host, etc. Under these biological constraints, it is expected that the connection between endoparasites and their hosts should be conservative to some extent in the course of endoparasite evolution. On the other hand, they have to explore and infect new host individuals by horizontal transmission, in order to survive and reproduce. During horizontal transmission they must frequently encounter various nonhost organisms, which may sometimes lead to the establishment of new endoparasite–host relationships. Therefore, the pattern of host specificity currently observed in a particular endoparasitic taxon is an evolutionary product of two components: maintenance of already established endoparasite–host connections (association by descent) and occasional host shifts (association by colonization) (Futuyma and Slatkin, 1983; Brooks and McLennan, 1991).

3. HOST RELATEDNESS HYPOTHESIS VERSUS HOST HABITAT HYPOTHESIS

To understand the evolution of endoparasite–host relationships, it is important to consider what factors have been involved in the acquisition of new hosts. In this context, two principal hypotheses have been formulated to address the factors that dominate the pattern of host shifts (Shaw, 1988). The host relatedness hypothesis suggests that host shifts tend to follow the host’s phylogenetic lines, on the grounds that related hosts will provide the most similar internal environment for endoparasitism. The host habitat hypothesis suggests that host shifts tend to follow the host’s microhabitat or feeding habitat lines, on the grounds that the probability of encounter can be a dominant factor in the endoparasite–host association. These hypotheses, though not necessarily mutually exclusive, are applicable to patterns of host shifts at different levels. When various endoparasitic taxa are examined for their host specificity, the pattern is universally found that a group of related endoparasites utilizes a group of related hosts, particularly at lower taxonomic levels. These cases favor the host relatedness hypothesis. On the other hand, at higher taxonomic levels of the endoparasites, it is frequently found that a group of related endoparasites utilize
distantly related organisms. To these cases the host relatedness hypothesis cannot be applied, but the host habitat hypothesis should be considered. As far as we know, however, although a number of reports have suggested the involvement of ecological overlaps based on obscure circumstantial evidence (Shaw, 1988; Klassen and Beverley-Burton, 1988; Durette-Desset et al., 1994), few studies have presented convincing phylogenetic analyses of host shifts that favor the host habitat hypothesis.

4. **DRAMATIC HOST SHIFTS BETWEEN ABSOLUTELY UNRELATED ORGANISMS**

Notably, dramatic host shifts between absolutely unrelated organisms have sometimes occurred in the evolutionary history of endoparasitic taxa. For example, in the trypanosomatid protozoans, most species are parasitic to vertebrates, but only one genus, *Phytomonas*, utilizes plants (Vickerman, 1994). In the endoparasitic bacteria of the genus *Wolbachia*, there are distinct but closely related lineages, some of which are parasitic to insects and others to arthropods, and still others are associated with filarial nematodes (O’Neill et al., 1997; Bandi et al., 1998). In the life cycle of endoparasitic protozoans and helminths, it is commonly found that phylogenetically unrelated hosts are utilized at different life stages (Gibson and Bray, 1994; Vickerman, 1994). Such remarkable “dramatic host shifts” must have expanded the host range, pioneered novel ecological niches, and had a great impact on the radiation and diversification in various endoparasitic groups. However, it is generally not easy to trace and reconstruct the evolutionary process of such dramatic host shifts. Because remarkable host jumping events usually occurred anciently in the history of the endoparasitic taxa, the status before and after the host jumping cannot be surely assigned based on the characters of extant organisms. Conversely, if detailed molecular phylogenetic analysis is conducted on an endoparasitic taxon in which a dramatic host shift occurred recently, we may be able to understand an important aspect of endoparasitic evolution.

5. **HOST ORGANISMS OF THE CORDYCEPS FUNGI AND PRESUMABLE HOST SHIFTS**

In these contexts, ascomycetous fungi of the genus *Cordyceps* are an interesting research subject. The *Cordyceps* is placed in the family Clavicipitaceae of the order Clavicipitales in the class Pyrenomycetes, whose members are known to be exclusively endoparasitic to insects (Mains, 1958; Kobayashi, 1982; Samson et al., 1988; Spatafora and Blackwell, 1993; Shimuzu, 1994). In general, members
of the Cordyceps show strict host specificity, although the degree of specificity differs from species to species. Some parasitize only a single host species (e.g., C. sobolifera strictly on the nymph of Platyleura kaempferi in Japan), while others utilize a range of hosts from a particular taxonomic group (e.g., C. militaris on the pupa of various moths).

Table 1 shows the host organisms of the Cordyceps and related entomoparasitic fungi. The majority of Cordyceps species utilize insect hosts from various orders such as Hemiptera, Lepidoptera, Coleoptera, Hymenoptera, Diptera, etc., suggesting that host shifts between insect orders have occurred in the genus. Some species parasitize noninsect arthropods such as spiders and mites, suggesting host shifts between the subphyla Chelicerata and Mandibulata in the Arthropoda. Notably, about 20 of the 400 described Cordyceps species are parasitic on hart’s truffles, hypogeous fungi of the genus Elaphomyces. Several species live on the sclerotium of plant-pathogenic clavicipitacean fungi, Claviceps spp. In addition, a few species are known to utilize the seeds of higher plants. Therefore, it is suggested that during the evolution of the Cordyceps fungi, interkingdom host jumping events must have occurred between the Animalia, Fungi, and Plantae.

6. MOLECULAR PHYLOGENETIC ANALYSIS OF THE CORDYCEPS FUNGI

In order to reconstruct the evolution of host specificity and the process of interkingdom host jumping, we investigated the phylogenetic relationships among 22 representatives, including 4 truffle parasites and 18 insect parasites, of the genus Cordyceps based on nuclear and mitochondrial rDNA sequences.

Information on fungal materials examined in this study is given in Table 2. Most of the Cordyceps species were collected in the field in Japan, except C. japonica [IFO9647] from the culture collection at the Institute for Fermentation, Osaka. The entomoparasitic deuteromycetes, B. bassiana [IFO4848], B. bronginartii [IFO5299], M. anisopliae [IFO5940], and P. tenuipes, are regarded as anamorphs of the Cordyceps spp. based on molecular phylogenetic and ecological lines of evidence (Shimazu et al., 1988; Liang et al., 1989; Fukatsu et al., 1997). H. chrysospermus [IFO6817] and H. lutea [IFO9061] were used as outgroup taxa.

All experimental procedures concerning DNA extraction, polymerase chain reaction (PCR), cloning, nucleotide sequencing, molecular phylogenetic analyses, and statistical analysis were conducted as described (Nikoh and Fukatsu, 2000).
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* According to the list of Shimizu (1994).

b In addition to Cordyceps, the list contains related entomoparasitic clavicipitacean genera such as Neocordyceps, Shimizuomyces, and Torrubiella, and their putative anamorphs such as Gibellula, Hymenostilbe, Hirsutella, Isaria, Paecilomyces, and Tilachlidiosps.

c Parasites of the sclerotium of Claviceps spp.

d Parasite of the seed of Campanumoea maximowiczii.

e Parasites of the seed of Smilax sieboldii.
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<sup>a</sup>This species is not yet described but is illustrated in Shimizu (1994) under the Japanese name “Amamisemitake” with species no.15.

<sup>b</sup>This species is not yet described but is morphologically similar to *C. pruinosa*. 

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7. MOLECULAR PHYLOGENETIC ANALYSIS BASED ON NUCLEAR rDNAs

The nuclear small subunit rDNA segment and the nuclear large subunit rDNA segment from the *Cordyceps* fungi were concatenated and subjected to phylogenetic analysis. Figure 1 is the neighbor-joining (NJ) tree based on an unambiguously aligned data set of 3014 nucleotide sites. Notably, four truffle parasites (*C. capitata*, *C. japonica*, *C. jezoensis*, and *C. ophioglossoides*) formed a monophyletic group, supported by 92.7% bootstrap value, with two cicada parasites (*C. inegoensis* and *C. paradoxa*). Hereafter, we call this monophyletic group “truffle-cicada clade.” Next to the truffle-cicada clade, six cicada parasites, a beetle parasite, a scale parasite, and a moth parasite constituted a poorly supported group. In this group, three cicada parasites

![Figure 1](image_url)

**Figure 1** Phyllogenetic relationship of the *Cordyceps* fungi based on nuclear rDNA sequences. A total of 3014 unambiguously aligned nucleotide sites were subjected to neighbor-joining analysis. Bootstrap values obtained with 1000 resamplings are shown at the nodes. Host organisms are presented in parentheses. Names of the clades are shown on the right side.
(C. kanzashiana, C. ramosopulvinata, and C. prolifica), two cicada parasites (C. sobolifera and C. sp. 1), and a scale parasite and a moth parasite (C. cocciidicola and C. cochlidiicola) formed monophyletic groups with nearly 100% bootstrap value. Hereafter, we call these clades “cicada clade A,” “cicada clade B,” and “scale moth clade,” respectively. In addition, there was a well-defined monophyletic group, supported by 100% bootstrap value, which was constituted by three moth parasites (C. militaris, C. sp. 2, and P. tenuipes) and two anamorphic generalists (B. bassiana and B. bronginartii). Hereafter, we call this monophyletic group “moth clade.”

8. MOLECULAR PHYLOGENETIC ANALYSIS BASED ON MITOCHONDRIAL rDNAs

Next, the mitochondrial small subunit rDNA sequences from the Cordyceps fungi were subjected to phylogenetic analysis. Figure 2 is the NJ tree based on an unambiguously aligned data set of 1258 nucleotide sites. As in the nuclear rDNA phylogeny, the truffle-cicada clade, supported by 89.0% bootstrap value, was identified in the mitochondrial rDNA phylogeny. Also, the cicada clade A, the cicada clade B, and the moth clade were supported by nearly 100% bootstrap values in the mitochondrial phylogeny. The scale moth clade was also identified, although the bootstrap support was low (68.8%). In conclusion, the nuclear rDNA phylogeny (Fig. 1) and mitochondrial rDNA phylogeny (Fig. 2) were reasonably concordant, although some discrepancies were found, particularly when statistical supports for the groupings were not significant.

9. PHYLOGENETIC RELATIONSHIPS OF THE CORDYCEPS FUNGI

To infer the intrageneric relationships of the closely related Cordyceps fungi as confidently as possible, the nuclear and mitochondrial rDNA data were combined to produce a large data set, which was subjected to detailed molecular phylogenetic analyses. Figure 3 is the strict consensus tree of three methods, NJ, maximum-likelihood quarter puzzling (ML-PUZZLE), and maximum parsimony (MP), based on an unambiguously aligned data set of 4272 nucleotide sites. As in Figs 1 and 2, the truffle-cicada clade, the cicada clade A, the cicada clade B, the scale moth clade, and the moth clade were identified with high bootstrap supports. In the truffle-cicada clade, C. japonica and C. jezoensis formed a monophyletic group supported by fairly high bootstrap values. In the moth clade, the relationships [C. militaris, P. tenuipes, (B. bassiana, B. bronginartii)] were supported with confidence. The other clades were, though appearing in Fig. 3, insignificantly supported.
In conclusion, two independent molecular phylogenies based on nuclear rDNAs (Fig. 1) and mitochondrial rDNA (Fig. 2) consistently supported these clades, indicating the reliability of the results. Based on the total sequence data of more than 4200 unambiguously aligned nucleotide sites, we present a phylogenetic tree of the *Cordyceps* fungi (Fig. 3) as a credible phylogenetic framework to understand the evolutionary aspects in this group.

10. EVOLUTION OF STROMATA MORPHOLOGY

At the maturity of the *Cordyceps* fungi, fruiting bodies or stromata of a spectacular shape grow out of the host insect. In their shape, size, and coloration,
in combination with the host species, the morphology of fruiting bodies shows a wide variety, although morphological characters are generally stable in a particular species (Kobayashi, 1982; Samson et al. 1988; Shimizu, 1994). When the shape of the fruiting body was arranged on the phylogenetic tree, it was evident that members of the same clade form fruiting bodies of similar shape (Fig. 3). Therefore, the five clades proposed by the molecular data were also supported by morphological characters, reinforcing the reliability of the phylogeny.

**Figure 3** Phylogenetic relationship of the *Cordyceps* fungi based on the total sequence data. A tandemly concatenated nuclear and mitochondrial rDNA data set (4272 nucleotide sites) was subjected to neighbor-joining (NJ), maximum-likelihood quartet puzzling (ML-PUZZLE) and maximum parsimony (MP) analyses. The strict consensus tree of the three analyses is presented. Numbers at the nodes are bootstrap values (%) obtained by the NJ (left), ML-PUZZLE (center), and MP (right) methods, respectively. Shown on the right side are host organisms, names of the clades, and morphological types of the stromata.
11. **EVOLUTION OF HOST SPECIFICITY**

In three of the five clades, the cicada clade A, the cicada clade B, and the moth clade, the members utilize hosts from the same insect group, which suggests that the endoparasite–host connections have been conserved to a considerable extent during the evolution of the *Cordyceps* fungi. These results favor the host relatedness hypothesis. The conservativeness of host specificity can be realized through two processes that are not necessarily incompatible (Futuyma and Slatkin, 1983; Brooks and McLennan, 1991). One process is co-speciation based on tight endoparasite–host connections. The other process is minor host shifts between phylogenetically related hosts based on relatively loose endoparasite–host connections. In the *Cordyceps*, cicada parasites generally parasitize only one or a few cicada species (Shimizu, 1994). In the cicada clades, therefore, the former process should be taken into account in addition to the latter process. On the other hand, it is known that moth parasites utilize a wide variety of lepidopteran larvae and pupae in general (Samson et al., 1988; Shimizu, 1994). In the moth clade, it seems quite likely that the latter process is overwhelming. To discuss these ideas with certainty, however, reliable information on the host range of the endoparasites and phylogenetic relationships of the hosts are required.

At the same time, Fig. 3 shows that major host shifts between distantly related host insects must have occurred repeatedly in the evolutionary course of the *Cordyceps*. In the scale moth clade, for example, it is suggested that a major host shift between Lepidoptera and Hemiptera took place. In this study, we examined 22 fungi whose host organisms included Hemiptera, Coleoptera, Lepidoptera, and *Elaphomyces* fungi. To date, 10 insect orders, Hemiptera, Lepidoptera, Coleoptera, Hymenoptera, Diptera, Odonata, Isoptera, Orthoptera, Blattaria, and Mantodea, have been recorded to be the hosts of *Cordyceps* species. In addition, some species parasitize noninsect arthropods such as spiders and mites. Furthermore, a small number of species have been known to live on other fungi such as *Elaphomyces* and *Claviceps*, and even on plant seeds (Table 1). Therefore, there appears to be no doubt that occasional major host shifts have expanded the host range, pioneered new ecological niches, and driven the diversification and speciation of the *Cordyceps* fungi. However, our results failed to reconstruct the evolutionary process of major host changes between insect orders with satisfactory resolution. Since parasites of spiders, mites, and plants were not included in this study, a number of drastic host shifts could not be investigated. More sequence data and more extensive sampling of *Cordyceps* species are necessary to analyze these aspects.
12. INTERKINGDOM HOST SHIFT FROM CICADA NYMPH TO HART’S TRUFFLE

The most important finding in the present study is the phylogenetic placement of truffle parasites in the *Cordyceps*. All four truffle parasites examined were placed in a well-supported monophyletic group, the truffle-cicada clade, together with two cicada parasites. Outside the clade were located a number of cicada parasites but no truffle-parasites. These phylogenetic relationships strongly suggest that (1) the ancestral host of the truffle-cicada clade is cicada, (2) hart’s truffle was acquired secondarily after the divergence of the truffle-cicada clade, and therefore (3) interkingdom host shift from cicada nymph to hart’s truffle must have occurred inside the clade. As far as we know, this is the first report that has definitively demonstrated the evolutionary process of interkingdom host shift in a single endoparasitic genus.

13. WHY DID THE HOST SHIFT FROM CICADA NYMPH TO HART’S TRUFFLE OCCUR?

Why from cicada nymph to hart’s truffle? What factors have promoted the jump between absolutely unrelated hosts? When we consider the life cycle and ecology of these organisms, a very interesting point in common emerges. Cicada nymphs feed on the xylem fluid of tree roots almost throughout their lifetime of several years underground (Yoshimura, 1997). Hart’s truffles are the mycorrhizal associate of tree roots and are completely subterranean throughout their life (Trappe, 1979). Their habitats are often as deep as around 10 cm under the ground, where few organisms as large as them are found. Although speculative, it is conceivable that the overlapping niches of cicada nymphs and hart’s truffles, both deep under the ground and associated with tree roots, may have promoted this drastic host shift (Fig. 4). We regard this finding as impressive evidence in favor of the host habitat hypothesis.

14. HOW MANY TIMES DID THE INTERKINGDOM HOST SHIFT OCCUR?

How many times have truffle parasites evolved from cicada parasites in the *Cordyceps*? Parsimoniously, a single origin is assumed. However, given the ecological factors that may facilitate the drastic host shift event, the possibility of multiple origins due to convergent evolution should be considered. To assess which of the alternative hypotheses is more likely, statistical analysis was conducted on the phylogenetic relationship in the truffle-cicada clade. The number of possible phylogenetic relationships of 6 fungi, 4 truffle parasites and 2 cicada parasites is 945. Log-likelihood scores for all the topologies were
FIGURE 4 Hypothesis: evolutionary process and promoting factors of interkingdom host shift from cicada nymph to hart's truffle in the genus *Cordyceps*.
calculated by the ML method based on the unambiguously aligned 4272 nucleotide sites of nuclear and mitochondrial rDNAs. Table 3 shows the five trees with the best log-likelihood scores. In the tree with the best score (Tree 1), the four truffle parasites were not monophyletic. Monophyly of the truffle parasites was found in the tree with the second best score (Tree 2). However, the difference in log-likelihood between them was only 0.3 ± 8.3, indicating that these topologies are almost equally likely. From these results, unfortunately, the monophyly of the truffle parasites was neither supported nor rejected because the relationship in the clade is too close to be resolved by the rDNA sequence data. Phylogenetic analysis using faster-evolving molecules will give an answer to this question.

15. WHEN DID THE INTERKINGDOM HOST SHIFT HAPPEN?

When did the drastic host shift happen? Berbee and Taylor (1993) analyzed the evolutionary history of radiation of the true fungi, in which the nucleotide substitution rate of fungal 18S rDNA was estimated to be around 1% per 100 MYA. By superimposing this rate on our data, the age of the host shift, presumably at the base of the truffle-cicada clade, was calculated to be 43 ± 13 MYA.

16. CONCLUDING REMARKS

Although drastic host changes must have occurred in various host–endoparasite systems, it is generally difficult to trace and reconstruct the evolutionary process in detail. The case of the Cordyceps fungi provides us with an opportunity to gain insights into how novel host–endoparasite relationships have been established.

<table>
<thead>
<tr>
<th>Tree no.</th>
<th>Topology</th>
<th>$l_l - l_{ML}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>(−9327.5)</td>
</tr>
<tr>
<td>2</td>
<td>(C1,(C2,M4,(M2,(M1,M3))))</td>
<td>−0.3 ± 8.3</td>
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<tr>
<td>3</td>
<td>(C1,(C2,(M4),(M2,(M1,M3))))</td>
<td>−3.7 ± 6.4</td>
</tr>
<tr>
<td>4</td>
<td>(C1,(C2,(M2,M4)),(M1,M3))</td>
<td>−8.6 ± 13.7</td>
</tr>
<tr>
<td>5</td>
<td>(C2,(C1,M4),(M2,(M1,M3)))</td>
<td>−9.2 ± 11.8</td>
</tr>
</tbody>
</table>

Table 3 Maximum Likelihood (ML) Analysis of the Truffle-Cicada Clade

- Of the 945 trees, the 5 trees with the best log-likelihood scores are shown.
- $C_1 = C$. paradoxa; $C_2 = C$. inegoensis; $M_1 = C$. jezoensis; $M_2 = C$. capitata; $M_3 = C$. japonica; $M_4 = C$. ophioglossoides.
- The log-likelihood of the ML tree is in parentheses.
ACKNOWLEDGMENTS

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The Evolutionary Strategy of *Claviceps*

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1. INTRODUCTION

Members of the genus *Claviceps* are specialized parasites of grasses, rushes, and sedges that specifically infect florets. The host reproductive organs are replaced with a sclerotium. However, it has been shown that after artificial inoculation, *C. purpurea* can grow and form sclerotia on stem meristems (Lewis, 1956) so that there is a capacity for epiphytic and endophytic growth. *C. phalaridis*, an Austrian endemite, colonizes whole plants of pooid hosts in a way similar to *Epichloë* and forms sclerotia in all florets of the infected plant, rendering it sterile (Walker, 1957, 1970).

To date, about 45 teleomorph species of *Claviceps* have been described, but presumably many species may exist only in anamorphic (sphacelial) stage and therefore go unnoticed. Although *C. purpurea* is the type species for the genus, it is in many aspects untypical, because most *Claviceps* species originate from tropical regions, colonize panicoid grasses, produce macroconidia and microconidia in their sphacelial stage, and are capable of microcyclic conidiation from macroconidia. Species on panicoid hosts with monogeneric to polygeneric host ranges predominate.
2. PHYLOGENETIC TREE

We compared sequences of ITS1-5.8S-ITS2 rDNA region for 19 species of *Claviceps*. Database sequences of *Myrothecium atroviride* (AJ302002) (outgroup for Bionectriaceae), *Epichloë amarillans* (L07141), *Atkinsonella hypoxylon* (U57405), and *Myriogenospora atramentosa* (U57407) were included to root the tree among other related genera. To the *Claviceps* species included in Pazůtová (2001), *Neoclaviceps monostipa* sequence was added (Sullivan et al., 2001) (sequence obtained courtesy of R. Sullivan) as well as four unpublished ones—*C. cynodontis* (Loveless, 1965), anamorphic *Claviceps* spp. *Erag* (from *Eragrostis* sp. Zimbabwe), Hyp (*Hypharrhenia rufa*, Zimbabwe), and UroPas (on *Urochloa* and *Paspalum*, Mexico). North American species *C. zizaniae*, already proved to belong to the *C. purpurea* group, was omitted from this data set because of long insertion and rearrangements in its ITS1 region.

Parsimony and the quartet puzzling tree with maximum-likelihood branch lengths were computed (Figs. 1 and 2). While other related genera were placed outside *Claviceps* clade, *N. monostipa* was firmly positioned inside the clade as sister species to *Claviceps phalaridis*. When the sequences were aligned, we found that certain *Claviceps* species have short ITS1, and the species with longer ITS1 filled that gap with entirely different sequences, probably as a result of independent events. Short ITS1 appears predominantly in species on more ancestral positions as in *C. paspali*, *C. citrina*, *C. cynodontis*, and *Claviceps* spp. SG and PM. Pair *C. phalaridis* and *N. monostipa* also had shorter ITS1. The differences in the neighboring sequences may be the result of repeated deletions, as is probably the case for *C. sorghicola*.

The quartet tree separated *Claviceps* species into the *C. purpurea* group, a group of tropical ergots, and *C. citrina*, whose relationship to the remaining groups was unresolved. On the most parsimonious tree, *C. citrina* was ancestral to the group of all tropical *Claviceps* species. No changes in the *C. purpurea* clade as compared to the tree in Pazůtová (2001) occurred by adding new taxa to the analysis.

*C. purpurea* has Palearctic distribution and colonizes pooids, arundinoids, and even panicoids. *C. paspali* originates from South America. *C. grohii* is a North American species from *Carex*, whereas *C. sulcata* (*Brachyaria* and *Urochloa*) is an African species and *C. fusiformis* (*Pennisetum* and *Cenchrus*) occurs in Africa and India (Loveless, 1967). *C. purpurea* and, as far as is known, *C. grohii* both lack microconidia and secondary conidiation. Despite different distributions, the similarity of rDNA sequences in this group is striking—at least 97% identity among *C. purpurea* and *C. grohii* was found.

Ergot species of tropical regions were divided into three clades. The first one contained African *Claviceps* sp. Hyp, *C. pusilla*, *C. cynodontis*, (both widespread in Old World tropics), and two South American anamorphic species,
**Claviceps** spp. SG (from *Setaria geniculata*) and PM (from *P. maximum*). Conidial morphology of the latter two isolates was not sufficiently related to described teleomorphic species to allow for their unequivocal association. *Claviceps* sp. Hyp was described by Loveless (1964b, 1985) on various *Hyparrhenia* species. A characteristic of this species is wide truncated macroconidia. Sclerotia are hidden in glumes, and their germination was never
achieved in laboratory. *C. pusilla* is easily recognizable due to its triangular macroconidia. Its host spectrum covers about 20 andropogonoid genera (e.g., *Bothriochloa*, *Cymbopogon*, *Capillipedium*, *Dichanthium*, *Heteropogon*, *Hyparrhenia*, *Vetiveria*, and *Themeda*) (Langdon, 1954; Loveless, 1964a, 1964b). Sequences of Hyp and *C. pusilla* were 97.6% identical. *C. cynodontis* has elongated to reniform macroconidia and it occurred in the Paleotropics. Its host plant, the chloridoid grass *Cynodon dactylon*, is believed to have originated in Turkey and Pakistan, and from there is was introduced to all tropical and subtropical regions of the world.

The second clade contained *C. phalaridis* (pooid parasite, endemic in Australia) and *N. monostipa* (panicoid parasite from Costa Rica). Surprisingly,
their rDNA sequences were 98.2% identical, although there are considerable morphological differences between these species.

The third clade was mostly unresolved. *C. africana*, and the Asian species *C. sorghicola* and *C. sorghi*, are all parasitic of *Sorghum*. *C. viridis* occurs on *Oplismenus* (Paniceae) in India (Padwick and Azmatullah, 1943; Thomas et al., 1945) and in Japan (Tanda, 1992). *C. gigantea* is specialized on the genus *Zea*. It represents so far the only parasite of andropogonoids that had to evolve in America from an ancestor introduced during the expansion of andropogonoid grasses. The sphacelial stage of *Claviceps* sp. UroPas was found by the author in Mexico (2000) as a parasite of *Urochloa* and *Paspalum*, and from *Claviceps* sp. Erag infecting *Eragrostis* sp. was collected by Frederickson in Zimbabwe (2001). Both these species cannot be assigned to any described teleomorph. To date, it has been assumed that the only species occurring on *Paspalum* is *C. paspali*. No teleomorphic species was recorded on *Eragrostis*.

3. HOST DISTRIBUTIONS

The distribution of ergot species among tribes of host grasses is unequal. There are considerable differences in the number of ergot species colonizing different subfamilies. The only chloridoid-specializing species known so far are *C. cynodontis* (*Cynodon*), *C. yanagawaensis* (*Zoysia*, Japan), *C. cinerea* (*Hilaria*, Mexico and southern United States), and *C. citrina* (*Distichlis spicata*, Mexico) (Pažoutová et al., 1998). This may be caused by the fact that chloridoid grasses often inhabit very dry habitats where *Claviceps* spp. are not able to survive. *C. cinerea* and *C. citrina* sclerotia develop in ascostromata 20–25 days after being placed on wet sand, without requiring months of dormancy (Griffiths, 1901; Pažoutová et al., 1998). This enables them to produce ascospores quickly after a rain period sets in.

Recently, however, arundinoid genera (*Phragmites*, *Molinia*, and *Danthonia*) were transferred to subfamily Chloridoideae (Grass Phylogeny Working Group, 2000) and *C. purpurea* and *C. phalaridis* occur on these genera.

The only parasites of pooid grasses are *C. purpurea* in Northern temperate regions and the endophytic *C. phalaridis*, endemic to Australia. As *C. purpurea* and *C. phalaridis* are quite distant species and despite that share the wide host range, it is probable that there is no barrier between genera of Pooideae that would prevent *Claviceps* species able to colonize one of them from spreading to all other genera; i.e., the wide host range may be caused by host metabolism and not by the parasite multiple adaptation.

*C. grohii*, *C. cyperi*, and *C. nigricans* colonize sedges and rushes in the Northern temperate regions.

Most species of the genus *Claviceps* are found on panicoid hosts. Their host specificity ranges from monogeneric (*C. paspali, C. viridis, C. gigantea*) to
polygeneric (C. fusiformis, C. pusilla). C. orthocladae, C. flavella, and C. diadema, all with primitive undifferentiated sclerotium encompassing the flower parts and often germinating directly on the host, are found on Orthoclada (Panicoideae, tribe Centothecae) and Panicum species in South America tropics. No such species were found in wet tropical and subtropical forests of Africa and South Asia.

Phylogeny of Claviceps does not mirror phylogeny of grasses. As the number of Claviceps sequences increases, there appears to be a certain tendency of the genus to fall apart into groups of closely related species, which may colonize quite unrelated host taxa (e.g., C. cynodontis from chloridoid grass related to Claviceps spp. PM and SG from Paniceae, or C. purpurea, C. sulcata, C. fusiformis, and C. grohii). One explanation for this may be the introduction of the group ancestor into a new area or ecological niche and subsequent colonization of the available host species with specialized populations from which later species arise. This may explain why C. viridis, specialized on Oplismenus, a C-3 panicoid grass with broad leaves (all primitive markers), is a close relative of Claviceps spp. colonizing andropogonoid grasses, which are the most advanced panicoid tribe. A similar strategy is now probably operating in C. purpurea and will be discussed later.

4. BIOGEOGRAPHY

The best-mapped distribution is that of Claviceps spp. occurring on cereals and pasture grasses, especially in Africa. Only a few species, C. diadema, C. flavella, and C. orthocladae, were observed in forest regions of the tropics and subtropics. These were collected in the nineteenth century in South America (Brazil, Guadeloupe, Cuba), and according to the original descriptions they possess primitive sclerotal characters or characters intermediate between Claviceps and Balansiae (Möller, 1901; Hennings, 1899; Diehl, 1950). No similar species was described from any other continent. Unfortunately, no recent collections of these fungi have been made that would enable more detailed studies.

Original distribution of Claviceps species in certain regions were especially well documented. Möller (1901) described Ascomycetes and Zygomycetes in the Brazilian state of Santa Catarina, in the region around Blumenau. He spent almost two years in 1891–1893 (he even founded a small local mycological society), and his book of 1901 is an interesting crossover of detailed observations with a kind of diary. He did not record the occurrence of any Claviceps species that would have resembled species from the Paleotropics, later described and/or revised by Langdon (1952) and Loveless (1964a, 1964b, 1965). Langdon, in his Ph.D. thesis (1952), described Australian Claviceps species which consisted of endemic species and Paleotropical ones. Moreover, he revised available herbarium specimens of most species known so far and corrected their
descriptions. It is a pity that this work was never published as a monograph. Loveless (1964–1985) spent about 20 years collecting and describing savannah Claviceps species in southern Africa, continuing the work of Doidge (1950). Except for C. maximensis, which he believed to have been introduced from Africa to South America with guinea grass, no common species between Neotropics and Paleotropics were found. As these detailed works precede the explosion of global seed and germplasm exchange, we may assume that Neotropical and Paleotropical Claviceps species were separated, and introductions of new species occurred probably only with host expansions.

In temperate regions of both hemispheres, C. purpurea prevails. Due to seed transfer by settlers, it is not possible to find out if the original distribution was in the Northern Hemisphere only. There is no other grass-colonizing Claviceps species. Related species are from sedges and rushes only, or in the case of C. zizaniae, an oryzoid host.

Original distribution of Claviceps species was affected during the last century by fodder grass seed and grain transfers. Examples are the spreading of P. maximum, C. dactylon (Loveless, 1964a, 1965), Brachiaria brizantha, Paspalum spp., and sorghum. C. paspali spread from South America to the United States around 1850, in the years 1927–1937 it reached Australia and New Zealand, in 1947–1948 the Mediterranean region, following the introduction of Paspalum distichum in 1929 (Hitchcock and Chase, 1950; Langdon, 1952). C. sulcata (Brachiaria spp.) was introduced from Africa to Brazil in 1995 (Fernandes et al., 1995). C. africana managed to affect sorghum-growing regions worldwide over 20 years (Bandyopadhyay et al., 1998; Pažoutová et al., 2000), and C. fusiformis (pearl millets in Africa and India) was recently found in Mexico on buffalo grass (Cenchrus) (San Martín et al., 1997). On the other hand, C. gigantea was never introduced outside Central America. C. cynodontis was originally distributed in the Paleotropics only. However, the author collected ergotized samples of Cynodon in Mexico (2000). Its conidial morphology and RAPD patterns were as similar to the ones of isolate from Zimbabwe as in two populations of the same species. Porter et al. (1974) described production of ergometrine and clavines in the cultures of the fungus isolated from ergotized C. dactylon (Mississippi), but no species identification was given. “Bermuda grass tremors” were reported in the United States in the 1950s. The origin of Claviceps sp. UroPas is uncertain. Although it was found in Mexico, its relatedness to Paleotropical species is remarkable.

5. ORIGIN

Langdon (1954) placed the origin of the genus Claviceps in the South America region formerly known as Gondwana. The first Claviceps species probably arose on the predecessors of panicoid grasses in the warm and humid climate of
Gondwana in the Upper Cretaceous. Our tree supports this hypothesis, as the ancestral species are predominantly from South and Central America. Moreover, the radiation center of panicoid grasses is in that region, and Claviceps species with primitive undifferentiated sclerotia were recorded in that area. Preservation of these lineages was probably facilitated by the isolation of South America from the end of the Cretaceous until the end of the Tertiary (Stebbins, 1981).

The first expansion of panicoid grasses in the early Tertiary probably gave rise to Claviceps species of the tropical clade. Further events influencing Claviceps evolution could have been the radiation of andropogonoid grasses from southern Asia to Africa, southern Europe, and Central America (Jones, 1991), reflected on the phylogenetic tree in the close relatedness of the Mexican maize parasite C. gigantea to Paleotropical andropogonoid parasites. Tropical Claviceps species are well adapted to semiarid conditions, but cold resistance enabling them to spread northward is limited.

The ancestors of species close to C. purpurea (relatives of C. paspali or C. citrina) might have migrated from South America to North America after the formation of the Panama land bridge and then to Europe and Africa. Only these species developed the ability to deal with cold winters and also with semiarid conditions. Sequence relatedness among the extant species of this clade occurring in the colder climatic regions and semiarid Africa suggests that the species diverged relatively recently.

The hypothesis of an early divergence between the C. purpurea group and tropical species is supported by the low or absent homology between DMAT synthase genes of both Claviceps clades. DMAT synthase is the first specific enzyme of alkaloid biosynthesis. This gene appears to be more variable than the ITS-rDNA region. Tudzynski et al. (1999) found 68% sequence similarity between the deduced sequence of the DMAT synthase protein of C. purpurea (CPD1) and its homolog DMAW from C. fusiformis (Tsai et al., 1995), whereas ITS-rDNA sequences of C. purpurea and C. fusiformis are 98.7% identical, and Rehner and Samuels (1995) observed 95.6% identity in a 960-bp fragment of 28S rDNA of C. purpurea and C. fusiformis.

Pazáutová (2001) hybridized digested genomic DNA of various Claviceps species with an 0.8-kb fragment of cpd1 gene. C. purpurea, C. fusiformis, C. sulcata, C. zizaniae, and C. grohii gave strong reactions, whereas more ancestral C. paspali with different ITS1 structure gave much weaker hybridization signals. Among tropical species, weak (C. africana, C. gigantea, and C. pusilla) or nonexistent DMAT signals in the DNA were observed, which suggests differences in secondary metabolism of both clades. C. africana (Mantle, 1968) and C. gigantea (Olsóská, 1999) produce alkaloids derived from the dihydroergoline skeleton, whereas ergoline alkaloids were found in the C. purpurea group. Moreover, there is evidence that the species from the tropical clade may produce nonergoline alkaloids. Bogo and Mantle (2000) found
caffeine in *C. sorghi* and *C. sorghicola*, which have not been shown to produce significant amounts of alkaloids of the conventional ergoline type.

6. MORPHOLOGICAL CHARACTERS

The taxonomic criteria used to delimitate *Claviceps* species are the color, size, and shape of sclerotia; the color of ascostromata (stipe and capitulum); the presence or absence of loose hyphae on the stroma; the size and shape of perithecia, asci, and ascospores (Langdon, 1942). From these markers, only sclerotium formation and type of asexual fructification bear phylogenetical importance.

Among both main clades, species with yellow, red-brown, or vinaceous to violet ascostromata are scattered without any tendency. On the *C. purpurea* clade, ancestral *C. paspali* belongs to the so-called yellow ergots, as does *C. sulcata*, whereas other species have red to dark red shades of coloration.

Perithecial dimensions, presence of septa in ascospores, and their length were also distributed among the species regardless of their position on the phylogenetic tree. From 21 species where the presence or absence of ascospore septa was mentioned in the description, 15 species had nonseptate, 5 had three- or multiseptate ascospores, and in *C. paspali*, 1 or no septum was recorded. Stipes of ascostromata are phototropic, so their length is to some extent influenced by the amount and direction of light available during germination. Size and shape of macroconidia is quite variable, mostly elongated and 10–15 × 3–5 μm. Only a few species are easily recognizable in the sphacelial/conidial stage of development: *C. pusilla* (triangular), Hyp (truncated), *C. rhynchelytri* (reniform), and *C. fusiformis* (allantoid) (Loveless, 1964b).

6.1. Sclerotium

Sclerotium size, and to some extent shape, is largely dependent on the space available inside the host floral cavity. For example, *C. purpurea* sclerotia produced in florets of *Poa annua* are about 1–2 mm long, those formed in florets of *Secale cereale* are up to 50 mm. Although sclerotia from wheat are more rounded than those from rye or common reed, they always protrude from the glumes so their shape differs from that of the healthy seed.

In sorghum ergot, *Claviceps africana*, the sclerotia were thought to be rounded, 3–5 mm in diameter, with reddish brown spots, until another population of this fungus specialized on *Hyparrhenia* spp. was discovered (D. Frederickson and S. Pažoutová, unpublished), whose sclerotia are cylindrical and brown to black, 2–4 mm in length, and 0.5–1 mm wide. Here, the sclerotium is shaped only by floral cavity, and even the color is influenced by the pigments supplied by the host. Sclerotia formed on sorghum cultivars with dark seeds are more
pigmented than those of yellow cultivars. These two examples document that morphology of sclerotium is not sufficient for species identification on different hosts.

The function of sclerotium is that of a dormant or resting structure. Its formation differs in *Claviceps* species. The simplest way is probably the proliferation of thick-walled cells accumulating lipids over the sphacelia, with cortex made up of layers of dead cells as demonstrated in *C. paspali* (Luttrell, 1977). In *C. sorghicola* (Tsukiboshi et al., 1999) and *C. citrina* (Pažoutová, unpublished), maturing sclerotium remains partially covered by a layer of conidiating sphacelial mycelium. In *C. gigantea*, sphacelia differentiates as a hollow structure and sclerotium arises inside this cavity as compact pale lavender-colored tissue covered with thin pigmented rind (Fuentes et al., 1964). The most organized sclerotium formation was observed in *C. purpurea* (Luttrell, 1980). Sclerotial and sphacelial differentiation are separated, and sclerotial apically directed intercalary growth starts in the proliferative zone distal to the sclerotial foot (site of contact with the plant vascular system).

Langdon (1954) described three types of sclerotia based on their development and resistance against climatic factors:

1. Primitive (balansioid), irregularly globose, where the mycelium emerges from the infected ovary and envelops parts of the spikelet(s) into pseudo-sclerotia or hypothallus resembling those of balansioid genera. Species producing these primitive sclerotial forms, *C. diadema* and *C. flavella*, occur in tropical regions.
2. Subglobose to elongated, usually light colored (*C. paspali, C. queenslandica, C. hirtella*), may contain remnants of floral parts.
3. Elongated sclerotia, ovoid to cylindric in shape, dark colored. On the distal tip of this sclerotium type, there is usually a cap formed by the remnants of sphacelial tissue. Species forming this type are found on members of all gramineous subfamilies. Their most advanced representative is *C. purpurea*.

### 6.2. Asexual Fructification

For the genus *Claviceps*, enteroblastic conidiation is typical. Phialides borne on short branched sporophores exhibit colarettes, and the conidia forming conidial heads remain attached by honeydew-like exudate. Branched sporophores (Fig. 3a) are *in planta* densely clustered on the surface of the young stroma, resembling palisade pseudo-parenchym. In vitro, conidiation occurs on short sporophores, occasionally branched, arising vertically from hyphae growing on the substrate surface. While macroconidia have different and often characteristic shapes, microconidia of all species forming them are rounded to oval, about 4–6 μm.
in diameter (Fig. 3b). Micro- and macroconidia may occur on neighboring sporophores (Pažoutová et al., 1977).

Most of the species also form single secondary conidia on germ tubes emerging from macroconidia (microcycle), either in honeydew drops or when plated on nutrient media (Fig. 3c). Their shape is oval or pearlike, always with a spiky proximal end, 10–15 μm in length, corresponding roughly to the macroconidium size. Microcycle is mostly finished in the course of 24 h after plating. Macroconidia of most species start colony growth in 5 days after secondary conidiation. Formation of secondary conidia is a good test of macroconidial viability. C. gigantea is exceptional in that its macroconidia die after completing microcycle, and colony growth is only achieved when explants of lavender tissue of the young sclerotium are plated.

In the descriptions from the end of the nineteenth and the beginning of the twentieth centuries, conidial size and mode of formation was often not sufficiently recorded or were even completely omitted. The authors were more focused on sexual structures and sclerotial characters, and even the host plant was not properly identified. Möller’s (1901) descriptions were an exception, because he not only described collected specimens but observed conidiation in vitro and documented it by excellent drawings. Diehl (1950) and Langdon (1952) revised

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**Figure 3** Asexual fructification in *Claviceps*. (a) Typical branched sporophores with protruding conidia. Conidia seen on the sporophores are invariably immature, as the mature conidia are easily detached in aqueous mounting media. (b) Universal shape of *Claviceps* microconidia. (c) Microcycle conidiation with pearlike and oval secondary conidia. (d) Ephelidial fructification of *C. citrina*. Conidia (3–7) are clustered on a whorl-like sporophore.

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descriptions of herbarium specimens for which Claviceps or Balansia affiliation was unclear, using conidiation type as criterion.

7. CLAVICIPITOID SPECIES WITH INTERMEDIARY CHARACTERS

There are a number of species with either primitive characters or characters intermediary between Balansiae and Claviceps. Some of the descriptions originate from the nineteenth century and are not easily available, therefore I will give them in details (Fig. 4).

7.1. Claviceps flavella

The type for Claviceps flavella was collected in 1869 in Cuba, and deposited in the Kew herbarium as related to Cordyceps. The host was not stated, the fungus being found among leaves. Petch (1933) noted that the sample contained “one sclerotium bearing five stalks and one and half detached heads.” Langdon (1952) described the Kew type sclerotium as brown, and enveloping the glumes, stipes and capitula were yellow, ostioles protruding.

![Figure 4](https://example.com/fig4.png)

**Figure 4**  
Claviceps flavella: (a) conidiophores on basal hypha; (b) sexual fructification; (c) perithecial head, arrow indicates collar. Claviceps diadema: (d) perithecial head; (e) chain of conidia and germination hyphae; (f) sclerotium germinating in planta.
Patouillard (1899) described a specimen collected by A. Duss in Guadeloupe as *C. pallida*. No identification of host was given. Samples were found on gramineous seeds, lying on the ground. Color of the head was whitish amber, with prominently protruding ostioles, stipe whitish, partially translucent. Perithecia were oval, 300 × 200 μm, ascospores nonseptated, up to 200 μm. Patouillard noted that the fungus does not produce true sclerotium, but grows on the “blackened seed tissue enveloped in glumes.”

Petch (1933) described other Duss specimens deposited in Herb. Berlin at that time. Here, mostly one clava was found on each sclerotium, with a stalk about 1 cm. The apex of the stalk was surrounded by a collar of about 0.25 mm. Sclerotia were seated on or surrounding the spikelet, with glume tips visible. Heads were dark brown, stalks pale red-brown, subtranslucent, sclerotia dark reddish brown with cortex red-brown in section.

Möller (1901) gave a detailed description of *Claviceps balansioides* from Brazil. The infection of host plant (*Echinochloa*) starts in the upper fertile floret, and later the lower sterile one and/or the whole spikelet are overgrown with hyphae. On the surface of glumes, a layer of mycelium appears, forming single elongated conidia of sphacelial type, 9–12 × 5 μm, without occurrence of conidial heads (Fig. 4a). Sporophores are thinner than normal hyphae. Later, mycelial mass fills the space available between the glumes in conical form, partially enclosing floral parts. The conidiating layer is replaced by dark blue-black rind. The irregular shape of sclerotium corresponds to the spikelet parts included; size depends on the amount of nutrition provided by the spikelet (Fig. 4b). The sclerotium was able to survive detached for several months. Only after that period did the germination into pale yellow perithecial heads occur. The stipes were unusually long, up to 8 cm, ending with collar (Fig. 4c).

*C. balansioides* was considered (Petch, 1933) to be the same as type *C. flavella* (Berk. & Curt). Diehl (1950) also transferred to this species *Claviceps pallida* (Pat.) from Guadaloupe. Sclerotial shape and structure for all the specimens is similar, however, Möller and Patouillard described it as black, whereas Petch observed reddish brown coloration. Stipes and capitula on the Kew, Möller’s, and Patouillard’s fungus were of yellow shades, whereas the ones on Petch’s samples were reddish brown, which Petch ascribes to storing.

### 7.2. *Claviceps diadema*

Möller (1901) first described *Claviceps diadema* as belonging to *Balansia* because of its loosely woven sclerotium, although the asexual fructification was more of sphacelial type (Fig. 4e). Therefore Diehl (1950) transferred this species into genus *Claviceps*. Möller observed that one or two neighboring *Panicum* spikelets were overgrown by hyphal mass. Structure of glumes was not destroyed
and still recognizable. Although this stroma was not hard as in, e.g., *C. purpurea*, it developed a dark yellow rind. Formation of anastomoses between the hyphae was frequently observed. The formation of 5–6 perithecial yellow heads with 2–4-mm stipes and very slightly protruding ostioles (Fig. 4d) occurred *in planta*, so that the germinating sclerotium resembled a little crown (hence the name) (Fig. 4f). *C. diadema* formed conidia that were 7–9 μm long, oval, tapering to the lower end, and Möller noted that they usually divide in two cells prior to germination. However, germination also occurred in conidia still attached to the conidiophore. Chains of conidia and germination hyphae were observed, but no formation of conidial heads glued together occurred. In the picture (Fig. 4b), they resemble more secondary conidia of other *Claviceps* species (Fig. 3c). Anastomoses occurred frequently between hyphae.

### 7.3. *Claviceps orthocladae*

*Claviceps orthocladae* (P. Henn.) Diehl (Orthoclada, Brazil), first described as *B. pallida* var. *orthocladae* (Hennings, 1900), also develops yellowish villous stipes and pale yellow capitula while its sclerotium is still on the host plant. Hennings (1904) merged this specimen with Möller’s *B. diadema* from *Panicum* and another Ule’s specimen (No. 1100), also from *Panicum* (Rio de Janeiro, Palmeiras), to a new taxon, *Balansiella orthocladae*. However, Diehl (1950) found in the original specimen sphacelial fructification only and placed this species into the genus *Claviceps*, unfortunately without revealing any details about conidial shape, size, and formation.

### 7.4. *Balansia pallida*

*Balansia pallida* (collected by Ule, 1885, Sao Francisco, Sta Catarina, Brazil, *Luziola peruviana*) (Winter, 1887) resembles *C. diadema*, and Hennings (1899) transferred it to the genus *Claviceps*. However, Winter observed that young stromata were covered with a fructification layer producing long (44–62-μm) curved conidia resembling ephelidial spores, which was the reason for Diehl (1950) returning the species to *Balansia* again. Diehl reexamined the specimen and found the hypothallus (0.5–2 mm in diameter) seated within florets of the host and partially enclosing the palea and lemma, with yellow surface and whitish interior. The yellow layer consisted of ephelidial fructifications. Ascostromata were yellow, with stipes 1–3 mm long, and heads 0.5–2 mm in diameter.

The more recent records of *Claviceps* species with “intermediary” characteristics are *C. phalaridis*, related *Neoclaviceps monostipa*, and according to yet unpublished observations, *C. citrina*.
7.5. **Neoclaviceps monostipa**

*Neoclaviceps monostipa* was found on panicoid grass in Costa Rica. It does not have true sclerotium; branching hyphae permeate the ovule tissues forming hypothallus. No host–parasite interface is established. From the hypothallus, a single reddish brown stipe (up to 3 mm) with capitulum emerges. The sequence of the ITS1-5.8SrDNA-ITS2 region of *N. monostipa* was 98.2% identical to that of *C. phalaridis* Walker, which is an Australian endemite. In cultures, *N. monostipa* produces conidia (36–72 × 1.2–1.6 μm) which undergo microcyclic conidiation partially resembling ephelidial fructification (Sullivan et al., 2001).

7.6. **Claviceps phalaridis**

*Claviceps phalaridis* persists as a systemic endophyte (Walker, 1957) in tillers, stems, leaf sheaths, and blades, similar to *Epichloë/Neotyphodium*, and forms sclerotia in florets of the diseased plants, rendering them sterile. It occurs on pooid grasses as well as on grasses of the chloridoid genus *Danthonia* (Walker, 1970). Sclerotium differentiation begins as a white fungal mass encompassing the anthers and ovary. Infected florets are later incorporated in the mature sclerotium. As distinct from *N. monostipa*, sclerotia of *C. phalaridis* are true resting structures with rind, however, in wet weather they are capable of germinating in planta. Ascostromata are of dark vinaceous color. Sphacelial fructification is almost nonexistent, and few oblong to cylindrical conidia (7.5–14 × 2–3 μm) were found on the sclerotial surface. Probably the same spores are formed during ascospore germination (Uecker, 1980). Each cell of the septate ascospore may give rise to one conidium borne on a short stalk. In addition to this other type of conidia, their shape, resembling those of *N. monostipa*, was observed (J. Walker, personal communication).

7.7. **Claviceps citrina**

*Claviceps citrina* was isolated and characterized in our laboratory from the sclerotia on the chloridoid grass *Distichlis spicata* (Mexico). Its sclerotia are true ones, readily germinating when placed in a humid chamber, in yellow ascostromata with stipes of 20–25 mm. In a previous publication (Pažoutová et al., 1998) we described only rounded to oval sphacelial conidia 4–6 μm in diameter on the surface of mature sclerotia collected in 1996. However, in the sample of younger sclerotia obtained in 1999, their proximal surface was partially covered with a white mycelial layer where, besides branched conidiophores of sphacelial type [Fig. 3a](#), producing oval conidia, long “setae” protruded. Recent examination revealed that these long spores are ephelidial conidia (47.5 < 59.3 < 70 μm × 1.5–2 μm) (Fig. 3d). On some sclerotia ephelidial
spores prevailed, on others the sphacelial ones. Among the remaining sclerotia from 1996 we also found two younger specimens containing both spore types. *C. citrina* appeared in our phylogenetical analysis as the most ancestral species.

8. ANCESTRAL CHARACTERS AND THE POSITION OF *CLAVICEPS* IN CLAVICIPITAE

Recent rDNA sequence analyses (Spatafora and Blackwell, 1993; Glenn et al., 1996; Kulda et al., 1997; Sullivan et al., 2001) confirmed monophyly of the Clavicipitales and their relatedness to the Hypocreales/Bionectriaceae. *Cordyceps* appeared in ancestral position to the plant parasitic Clavicipitaceae. The clades depicting groupings of genera of plant parasitic Clavicipitaceae have low statistical support. If *C. citrina* sequence is compared with the Gen Bank database using a BLAST algorithm, the most related species are (in order of decreasing similarity) *C. phalaridis*, *Myriogenospora atramentosa*, *Balansia strangulans*, *E. amarillans*, and various other *Epichloe* spp. The next *Claviceps* species appears as 11th. On our trees, the clade separating *Claviceps* from other genera has low or no statistical support; only the clades separating groups of related species are supported.

Not only sequences of Clavicipitaceae but also morphological characters gave unequivocal information. Anamorphs with holoblastic (ephelidial) conidiation and enteroblastic (sphacelial, typhodial) conidiation are encountered, as well as species and genera with synanamorphs of both types.

If we consider some *Cordyceps* characteristic (enteroblastic phialidic conidiation, stipitate hermispherical ascostroma, formation of hard resting stroma), then all these are also found in the genus *Claviceps*. Specific characters relating to plant parasitism are colonization of florets (more or less specialized) with a potential for colonization of meristems (as demonstrated by *C. purpurea* inoculation experiments). Production of slime gluing together conidial heads is increased to such an extent that honeydew with suspended conidia drops outside the florets. Among *Claviceps* species on ancestral positions, characters typical for other clavicipitoid genera are found, such as ephelidial conidia and endophytism.

Plant parasitic clavicipitoid genera might have arisen from variable *Claviceps*-like ancestors, as no other genus has extant species sharing so many characters with other related genera as *Claviceps*. Such ancestor might have had stipitate ascostromata, loose sclerotium of *C. flavella* type encompassing flower parts without establishing fungus/host interface on vascular bundle. Some of these early species might have occurred as occasional endophytes. The existence of synanamorphs of phialidic and ephelidial type as in *C. citrina* is probable, *Ephelis* anamorph being derived character.
9. POPULATIONS AND SPECIATION MECHANISMS IN CLAVICEPS

9.1. Claviceps purpurea

*Claviceps purpurea* is an ergot fungus with a wide host range including the entire subfamily Pooideae and also genera belonging to chloridoids and panicoids (Loveless, 1971; Brewer and Loveless, 1977). Its distribution is basically Holarctic, but it has been recorded in Arctic regions (Linder, 1948) and also occurs in southern temperate and subtropical regions.

Most attempts at establishing host-specific populations or subspecies taxa such as varieties, special forms, or races was made on *C. purpurea*, probably because of its wide host range and also greater accessibility of different isolates and collections. It can be argued now that the range is not the character of *C. purpurea* but that of pooid grasses, as *C. phalaridis* is also able to colonize various pooid hosts, even the genera introduced to Australia.

Morphology of *C. purpurea* is variable. Sclerotial length ranges from 2 to 50 mm and the color of the stromata varies over a wide scale of red shades. Conidial size and shape also are polymorphic, ranging from oval spores 5 μm in length to cylindric or elongated and up to 13 μm in length (Loveless, 1971; Sprague, 1950; Tanda, 1979). The sclerotia contain peptide alkaloids that belong to three basic groups: ergotamines (with alanine as the first amino acid entering the cyclopeptide moiety), ergytoxines (with valine), and rarely found ergoxines (with 2-aminoisobutyric acid) (Walzel et al., 1997).

In the herbaria, *C. purpurea* specimens can often be found under the names of *C. wilsonii* (used for ergot from *Glyceria fluitans*; Barger, 1931), *C. microcephala* (samples from *Poa annua*), or *C. sesleriae*. There were misidentifications of *C. fusiformis* as *C. microcephala* (Thirumalachar, 1945) persisting until the 1970s (Sundaram et al., 1972).

Historical overviews of races and varieties of *C. purpurea* introduced by different authors are given by Barger (1931), Loveless (1971), and Pažoutová and Parbery (1998). Especially, Stäger (1903, 1908) defined several races, which were subsequently modified and rearranged, however one of them went almost unnoticed. Stäger (1922) observed that sclerotia formed on grasses from wet habitats could float on water, but that sclerotia from *Secale, Lolium, Brachypodium sylvaticum, Sesleria coerulea, Arrhenatherum elatius, Agropyron* (now *Elyttrigia*) *repens, Alopecurus myosuroides*, and other land grasses sank in water. On *Dactylis glomerata, Calamagrostis epigeios*, as well as some *Holcus* and *Poa* spp., sclerotia of both types were found. Stäger named that race f. sp. *Phalaridis arundinaceae natans*. Defining this taxon based on habitat was against the host-based system of his other races, so unfortunately this line of research was discontinued.
Loveless (1971) found that the conidia of isolates from grasses from wet/shady habitats were longer (6.5–8.5 µm) than those from isolates found on land grasses (5–6 µm), and the spores from laboratory cultures showed more variation than the ones from natural hosts. Grouping of specimens according to conidial size and host corresponded partially to Stäger’s groups.

Kobel and Sanglier (1978) found 10 chemoraces in sclerotia collected in Europe and North America, the most usual combinations being ergocornine/ergocryptine (22.6% of samples), ergocristine/ergosine (20.4%), and ergotamine (13.1%). Composition of the alkaloid mixture produced is hereditary and independent of host grass (Kybal and Brejcha, 1955).

No attempt was made to compare the alkaloid races with host-based grouping except for a Czech study of natural occurrence of chemoraces (Kybal et al., 1957) together with characterization of host and location. Seventeen sclerotal specimens (of 32) were of ergocristine/ergosine type, found on Phalaroides arundinacea, Calamagrostis spp., Holcus spp., Molinia spp., Festuca rubra, Festuca gigantea, D. glomerata, and Phragmites communis growing in wet or forest locations. Thirteen specimens occurring on Hordelymus europaeus, Elytrigia repens, F. pratensis, H. lanatus, and S. cereale growing on meadows contained mixtures of ergosine, ergocornine, and ergocristine. Only two specimens produced small amounts of ergotamine in addition to ergocornine and ergocryptine, and both originated from Lolium growing along the roads.

Another group of C. purpurea isolates was found on Spartina spp. populating salt marshes of the Atlantic shore in the Americas. This group was characterized (as analyzed by thin-layer chromatography) by predominant production of ergocryptine, ergocryptinine, and lysergylvalylmethylester (Eleuterius and Meyers, 1974). Spartina stands in the British Isles were colonized by C. purpurea only after 1960 (Hubbard, 1970; Raybould et al., 1998). These isolates have the longest conidia (8.4 µm) of all the British samples studied (Loveless, 1971).

Jungehülsing and Tudzynski (1997) established two main groups using RAPD typing: one consisted mainly of the English isolates from Molinia, Holcus, and Dactylis, the other group contained the isolates from land grasses.

Our study (Pažoutová et al., 2000) established the population structure of C. purpurea and characterized the groups and isolates by host or habitat preferences, phenotypic traits used in previous studies (conidial morphology, alkaloid type, properties of sclerotia), as well as by DNA analysis (using RAPD and EcoRI restriction site polymorphism in the 5.8S rDNA). Thus, the ambiguous, even contradictory groupings found by previous researchers and based on only one or two characters were incorporated into one system. Three groups were identified:
G1, from fields and open meadows
G2, from shady or wet habitats
G3, from Spartina salt marshes

The sclerotia of G1 contained various ergotamines and ergotoxines; its conidia were 5–8 μm long. G2 produced ergosine and ergocristine with small amounts of ergocryptine; conidia were 7–10 μm long. G3 produced ergocristine and ergocryptine, and conidial length was 10–12 μm. Sclerotia of the G2 and G3 isolates floated on water. In the 5.8S rDNA, an EcoRI site was found in G1 and G3 but not in G2.

Typical hosts of G1 were S. cereale, Lolium spp., E. repens, F. pratensis, Helictotrichon pubescens, and Bromus spp.

Isolates of G2 were more commonly recovered from Calamagrostis, Holcus, Molinia, Phalaroides, and Phragmites growing at ponds and riverbanks, ditches, forests, mountain woods.

Alopecurus pratensis, Ammophila arenaria, Arrhenatherum elatior, Dactylis sp., Festuca ovina, F. rubra, Phleum sp., and Poa pratensis could be naturally colonized by isolates of both G1 and G2. The habitat seems to be more important, as the common occurrence of different groups in the same locality is rare.

The third group, G3, was found on Spartina alterniflora (introduced from North America) and S. anglica stands in coastal salt marshes in Wales, Essex, and Yorkshire, and at four locations in the Southampton region. RAPD profiles of American and British isolates from these grasses were uniform (Pažoutová et al., 2002), suggesting transfer of the G3 group from the American Atlantic coast to Europe. Mass ergot infection on S. anglica and S. alterniflora by C. purpurea with unusually long conidia appeared first in the 1960s (Loveless, 1971; Raybould, 1998).

To some degree, host preferences were observed. Our preliminary inoculation experiments using spraying of florets with conidial suspension showed that host species typical for the given habitat (such as Phragmites or Phalaroides) are only weakly colonized by C. purpurea isolates from different habitats (those belonging to G1 or G3). Host genera common to both the G1 and G2 (especially P. pratensis and Dactylis spp.) are grasses which may be encountered in either habitat. These preferences result from adaptation to available hosts.

C. purpurea isolates were analyzed using RAPD and AFLP (Pažoutová et al., 2000; Pažoutová et al., 2002). The difference between populations were considerable, as both methods detected only 1–3 bands shared between populations. Together with comparison of rDNA (ITS1-5.8S-ITS2 region), all three methods suggest that the G1 population is ancestral to the G3 and G2, which are more homogenous.
A possible cause for increased intraspecific variation in *C. purpurea* might be chromosomal rearrangements. Hüsgen et al. (1999) observed variations in the chromosome number and size in a set of *C. purpurea* isolates. We have typed some of their isolates that were part of the set analyzed by Jungehülsing and Tudzynski (1997) to our G1 or G2 groups. Different karyotypes and ploidy were found across the groups. Two triploids were found, both from the G2 group, and the most of the isolates were diploids or aneuploids. Only 4 of 23 isolates were haploid.

The G2 and G3 groups also share some phenotypical similarities such as elongated or cylindrical conidia, floating sclerotia, and ergocristine as one of major alkaloids. Therefore it may be inferred that a group of isolates with floating sclerotia arose from ancestors of extant group G1 which later diverged into G3 and G2 (the latter lost in addition a conserved EcoRI site in 5.8S rDNA). Indeed, we found such an isolate with RAPD and alkaloid composition (ergosine, ergocornine, ergocryptine) typical for G1, however, its sclerotia floated and it was found on the water grass *Glyceria fluitans*.

Interestingly, similar habitat association was recently discovered in an insect pathogen, *Metarrhizium anisopliae*, where forest and field populations were found differing in UV and cold tolerance and no host specificity was found (Bidochka et al., 2001).

### 9.2. *Claviceps africana*

*Claviceps africana* spreads mainly via secondary conidia. Formation of secondary conidia from primary macroconidia is widespread, especially among tropical *Claviceps* species, however, we observed that this process occurs either inside honeydew drops or in vitro when primary macroconidia are plated on agar medium. In *C. africana*, secondary conidiation occurs on macroconidia close to the surface of honeydew drop (Frederickson et al., 1989). Resulting conidia form a dry layer outside the drop and are spread by wind (Frederickson et al., 1993). This strategy differs from the usual spreading through insect vectors attracted by sweet honeydew and enables long-distance jumps.

The importance of ascostroma formation in population variability is unclear. Most sphacelia formed in sorghum fields do not mature in sclerotia. Germination of sclerotia in vitro or observed in the field was erratic (Frederickson et al., 1991).

Recent spreading of *C. africana* from Africa to sorghum-growing regions worldwide prompted studies about its internal variation and population structure (Pažoutová et al., 2000a; Tooley et al., 2000). RAPD and AFLP were used, and both methods revealed only very small differences between isolates, confirming homogeneity of sorghum populations. American isolates were almost identical and three isolates of the same type were also found in South Africa, suggesting
the origin of the invasion clone in the Americas (West group) first recorded in Brazil in 1995 (Reis, 1996).

In India, sorghum ergot was caused by endemic *C. sorghi*. Although conidia of this species are indistinguishable from those of *C. africana*, no wind-borne conidia are formed; its sclerotia protrude from glumes and are easier to germinate. Early observations in 1914–1917, 1946 (Ramakrishnan, 1948), and during the early 1960s (Singh, 1964) mention mostly large elongated sclerotia found on sorghum. Both long and short sclerotia were found in the late 1970s (Sangitrao and Bade, 1979) to mid-1980s, which might suggest the start of *C. africana* infections. Since the late 1980s, the typical long and protruding sclerotia of *C. sorghi* have not been observed.

DNA typing studies confirmed *C. africana* replacing *C. sorghi* (Pažoutová et al., 2000a; Tooley et al., 2000). The population was different from that invading the Americas. Despite all quarantine precautions it reached Australia in 1996 (Ryley et al., 1996). Inside that population (East), Australian isolates were distinguishable from India ones by acquisition of single RAPD band difference, which also appeared in an isolate from Thailand. This suggests an infection path to Australia through Southeast Asia islands.

RAPD markers of the East and West groups behaved like bar codes—the isolates lacked or shared them all. However, in African isolates, RAPD markers appear like independent characters and occur in combinations. Still, the sorghum population is very homogenous.

Recently, a *Claviceps* sp. on *Hyparrhenia rufa* was found in southern Africa (Frederickson and Pažoutová, unpublished) whose conidia were very similar to *C. africana*, and its sclerotia were of the same shape as seeds of the host plant. RAPD patterns showed that it is indeed another population of *C. africana*, but more different than any of the sorghum isolates, which may be the beginning of speciation based on host adaptation. Although *Hyparrhenia* plants were neighbors of a sorghum field, no RAPD pattern similar to that of *Hyparrhenia* population was found among sorghum isolates.

9.3. **Claviceps gigantea**

In 1996 we collected in Central Mexico about 30 isolates of *C. gigantea* (Fuentes et al., 1964) from five locations in the region of Toluca (*Zea mays*) and from a location near Amecameca (*Z. mays* and *Z. mexicana*) (Pažoutová and Fučíkovský, unpublished). Sclerotia of *C. gigantea* remain in the field, and those harvested with corn are easily discarded due to their size so that no transfer with seeds occurs. As the sphacelia is completely wrapped in husks, no honeydew with conidia is available for insects to spread. Therefore differences between populations isolated in the small valleys were expected. However, although the locations were isolated from each other by mountain ridges, neither RAPD with
50 primers nor AFLP with three primer pairs detected any reproducible differences, not even between the Toluca Valley and Amecameca isolates. *C. gigantea* did not spread from Central America to maize-growing regions worldwide because it has specific temperature and humidity requirements that are met especially in high valleys of central Mexico (Fučíkovský and Moreno, 1971).

The role of sexuality in the speciation process is unclear. *C. purpurea* was proved to be homothallic, which should (similarly to asexual reproduction) support separation of the population in clonal lineages. Small variability of *C. gigantea* and *C. africana* shows that this is not occurring. On the other hand, no isolates that might be hybrids of ecoraces have been found in *C. purpurea* so far, maybe due to effective separation of the populations, which rarely occur on the same locality.

From these three analyses it may be hypothesized that *Claviceps* species infraspecific variation remains low in stable environments, as was observed in *C. gigantea* adapted to mountain climate and in habitat-specialized ecoraces G2 and G3 of *C. purpurea*. *C. africana* shows small but detectable variability of sorghum isolates, however, RAPD pattern of *Hyparrhenia* isolate differs considerably with all primers used. It seems that strong external impulses such as change of host or transfer into another environment trigger population variability and lead to speciation, aimed at the ecological niches available. Recent transfers of ergot fungi are precisely the kind of events that stimulated their evolution so far, so the adaptation of introduced species to new host genera and further spreading is to be expected.

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1. INTRODUCTION

Fungi are by far one of the most important sources for biologically active substances, and a number of drugs derived from fungal secondary metabolites and their modified analogs have been developed. Among a wide variety of fungi, the Clavicipitaceae are one of the frequently explored families of Pyrenomycetes. Among the most important discoveries of bioactive secondary metabolites from fungi may be the isolation of the β-lactam antibiotic, cephalosporin C, from *Cephalosporium Acremonium* in 1948 (Sec. 7). Cyclosporin A, originally found from *Tolypocladium polysporum* in 1976, and its analogs, are now commonly used as immunosuppressants (Sec. 6.1). Much earlier, a series of alkaloids produced by the plant pathogenic species *Claviceps purpurea* (“ergot”) were recognized to cause serious toxic effects to animals (including humans) that ingested infected material. This condition is commonly referred to as ergotism and has been reported...
in the literature since medieval times. This fungus and its metabolites have been thoroughly explored (Sec. 10.1). On the other hand, an insect-host specific fungus, *Cordyceps sinensis* (Dong-Zhong-Chang-Cao), has long been used as a traditional medicine in China (Sec. 12). Important secondary metabolites isolated originally from Clavicipitalean fungi are reviewed in this chapter. Due to the frequent intergeneric overlapping of compound groups, this chapter is organized by chemical types rather than fungal genera.

2. TERPENES

Several types of novel terpenes have been isolated from Clavicipitaean fungi (Fig. 1). The plant pathogenic fungus *Epichloë typhina* produces structurally simple fungi-toxic cyclopentanoid sesquiterpenes, chokols, such as chokols A (1), E (2), and F (3) (Yoshihara et al., 1985; Koshino et al., 1989a). *Acremonium strictum* produces a GABA-benzodiazepine receptor-binding sesquiterpene, xenovulene (4) (Ainsworth et al., 1995). Xenovulene inhibited flunitrazepam binding to the GABA-benzodiazepine receptor with an IC50 of 40 nM in an in-vitro assay using bovine synaptosome membrane preparations. Aphidicolin (5), a diterpene inhibitor of DNA polymerase α, was isolated from *Cephalosporium aphidicola* IMI 68689 (Dalziel et al., 1973). Deoxynortrichoharzin (6), isolated from a sponge-derived *Paecilomyces* cf. *javanica* (Rahbæk et al., 1998), is a new analog of trichoharzin diterpenes. Cephalonic acid (7), isolated from *Cephalosporium caerulens*, exhibits weak activity against *Staphylococcus aureus* (Itai et al., 1967). Sesterterpene (8), from *Paecilomyces inflatus*, is reported to be a very potent glycosylphosphatidylinositol (GPI) inhibitor (Wang et al., 1998). Thus, this compound inhibits GPI synthesis in vitro of yeast microsomes with a MIC of 3.4 nM.

A new triterpene, 3β, 15α, 22-trihydroxyhopane (9), together with a known dustanin (15α, 22-hydroxyhopane), were isolated from *Aschersonia aleurodis* CBS 541.73 (van Eijk et al., 1986), while a new analog (10), along with (9) and dustanin, were reported from *Aschersonia tubulata* BCC 1785 (Boonphong et al., 2001). Interestingly, compounds (9) and (10) exhibited activity against *Mycobacterium tuberculosis* R37a with MIC of 12.5 μg/mL. Systematic analyses of the secondary metabolites of insect-associated species of Clavicipitaceae revealed that dustanin and a related known triterpene, zeorin (6α, 22-dihydroxyhopane), have been commonly found in many species of the genus *Aschersonia* but not from other insect pathogenic fungi (Kittakoop and Isaka, unpublished).

Long-chain terpenes, SCH 60065 (11) and nine other related analogs, have been isolated from *Acremonium* sp. (Hedge et al., 1997). These compounds are neurokinin (NK) receptor inhibitors with IC50 values ranges of 2.5–11 μM (NK1) and 6.8–16 μM (NK2).
FIGURE 1 Terpenoids.
3. STEROLS

There have been few reports of new steroids from Clavicipitalean fungi (Fig. 2). An endoperoxide (12) and an epoxy-sterol (13) were isolated from *Cordyceps sinensis* (Bok et al., 1999). A ring B aromatic steroid (14) was reported to be among constituents in the stromata of *Epichloe typhina* growing within *Phleum pratense* (Koshino et al., 1989b). Four new steroids (15–18) were isolated from *Verticillium lecanii* (Claydon et al., 1984; Grove, 1984a, 1984b). *Tolypocladium koningii* and a mutant strain of *Tolypocladium inflatum* DSM 915 produced highly oxygenated steroids, ergokonins A (19), B (20), and C (21) (Gräfe et al., 1991). Ergokonins are antifungal compounds displaying strong activity against *Candida* species.

![Sterols](image-url)
4. POLYKETIDES

4.1. Nonaromatic Polyketides and Substituted Benzenes

Cephalosporolides A-G (e.g. 22) are a group of 10-membered macrolides isolated from *Cephalosporium aphidicola* (Ackland et al., 1985; Farooq et al., 1995) (Fig. 3). From the same fungus, a dimer with a thioether linkage,

![Cephalosporolides A-G](image)

*Fig. 3* Polyketides (nonaromatic, and substituted benzenes).
thiobiscephalosporolide (23), was also found (Ackland et al., 1984). A spirocyclic lactone, paecilospirone (24) from Paecilomyces sp. was identified (Hirota et al., 1991). Two δ-lactones, gamahonolides A (25) and B (26), isolated from the plant pathogenic fungus Epichloë typhina on Phleum pratense, exhibited weak antifungal activity (Koshino et al., 1992a). Acremonium roseum I4267 produces a novel γ-lactone, acremolactone A (27), which exhibits potent...
herbicidal activity (Sassa et al., 1998). Acremolactone A is especially active against harmful weeds, particularly notable is its preemergence herbicidal activity against crabgrass (*Digitaria adscendens* Henr.) and smartweed (*Polygonum blumei* Meisn.), which was rated in the range 9–10 on a 0–10 (complete killing) rating system at the dosage of 1.0 kg/ha.

F-11334s (28–32), isolated from *Acremonium murorum* SANK 20793, are reported as inhibitors of membrane-bound neutral sphingomyelinase (N-Smase). IC$_{50}$ values of these compounds on N-Smase of rat brain microsome fraction under neutral conditions were 7.5, >200, >200, 3.6, and 3.2 µg/mL, respectively, suggesting that free para-hydroxyl groups in the hydroquinone moiety were essential for enzyme inhibitory activity (Tanaka et al., 1999). Two new hydroxymethylphenols, bigutol (33) and methylbigutol (34), isolated from the mycoparasite *Verticillium biguttatum*, exhibit moderate antifungal activity against a plant pathogen *Rhizoctonia solani* (MIC 138 µg/mL) (Morris et al., 1995). A culture filtrate of *Verticillium albo-atrum* contained a structurally simple yet novel phytotoxin, named alboatrin (35). At a concentration of 50 ppm, this compound inhibited the root growth of the host plant (Maris Kabul) at 49% (Ichihara et al., 1988). PS-990 (36) from *Acremonium* sp. KY 12702 inhibited brain calcium/calmodulin-dependent cyclic neurotide phosphodiesterase with an IC$_{50}$ value of 3 µg/mL, and markedly induced neurite extension of mouse neuroblastoma, Neuro2A, at concentrations ranging from 10 to 30 µg/mL (Toki et al., 1994). CRM646-A (37) and -B (38), isolated from *Acremonium* sp. MT70646, are novel heparinase inhibitors with IC$_{50}$ values of 3 and 10 µg/mL, respectively. In an invasion assay of B16-F10 melanoma cells through laminin-coated matrigel, compounds (37) and (38) showed inhibition of B16–F10 cell migration (IC$_{50}$ 15 and 30 µM, respectively), with negligible cytotoxic activity against various cell lines at concentrations up to 100 µM. The above results indicated that these compounds are potential new candidates for use in the study of metastasis and angiogenesis (Ko et al., 2000). A series of biosynthetic related compounds (39–41) has been isolated from *Verticillium intertextum*. Together with vertinolide (39), 2′, 3′-dihydrosorbicillin (40) and two known sorbicillin analogs, a dimer, bisvertinoquinol (41) was isolated (Trifonov et al., 1981, 1983). Apart from these, which have been described, four dimeric vertinolides, bisvertinols (42–45), were later found from the same fungus (Trifonov et al., 1986).

4.2. Polycyclic Aromatics (Pigments)

Many pigments, bearing naphthol, naphthoquinone, oxanthracene, anthraquinone, or xanthone moieties, either as monomers or more commonly as dimers, have been found in Clavicipitalean fungi, particularly from the genera *Cordyceps*, *Paecilomyces*, and *Verticillium* (Fig. 4).
Six naphthoquinone derivatives, including two new compounds, 4-O-methyl erythrostone (46) and compound (47), were isolated as antimalarial and cytotoxic constituents of Cordyceps unilateralis BCC 1869 (Kittakoop et al., 1999). Compound (46) and four known erythrostonees exhibited in-vitro antimalarial activity against Plasmodium falciparum K1 (IC$_{50}$ 4.0–10 µg/mL).

**Figure 4** Polyketides.

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and cytotoxicity against two cancer cell lines (BC-1, IC$_{50}$ 4.2–10 μg/mL; KB, IC$_{50}$ 7.2–24 μg/mL) and Vero cells (African Green Monkey kidney fibroblast cells) (IC$_{50}$ 7.5–30 μg/mL). In contrast, compound (47) strongly inhibited proliferation of the malaria parasite (IC$_{50}$ 2.5 μg/mL), but was not cytotoxic to the three cell lines up to a concentration of 50 μg/mL.

From a fermentation broth of Spicaria divaricata NRRL 5771 (identical to Paecilomyces variotti) collected from air in the laboratory, produced two new
naphthopyrane dimers, SC-28763 (48) and SC-30532 (49) (Jiu and Mizuba, 1974; Mizuba et al., 1977), together with viriditoxin (R1 = R2 = OCH3). The latter was first reported as a product from Aspergillus viridinutans (Plectomycetes). These compounds exhibited moderate antifungal activity against Clostridium perfringens ATCC 13124 and Trichomonas vaginalis ATCC 30001. Although structures of compounds (48), (49), and viriditoxin were presented as C8–C8’ dimers, this was later revised to the C6–C6’ dimers as depicted (Suzuki et al., 1990). Corresponding monomers, semiviriditoxin (50) and semiviriditoxic acid (51), were isolated as new natural products from Paecilomyces variotii NOF-1232 (Ayer et al., 1991).

Six new anthraquinones, paeciloquinones A-F (52–57), were isolated from Paecilomyces carneus P-177 (Fredenhagen et al., 1995). Paeciloquinones are reported to be potent protein tyrosine kinase inhibitors (Petersen et al., 1995).

Eight bioxanthracenes, ES-242s (e.g. 58–63), were isolated from Verticillium sp. SPC-15898 (Toki et al., 1992a, 1992b). ES-242s are antagonist of N-methyl-d-aspartate (NMDA) receptor, inhibiting [3H]thienyl cyclohexyl-piperidine binding to rat crude synaptic membranes. The IC50 value of the most potent compound, ES-242-1 (62), was 0.116 μM, while other ES-242s showed much weaker inhibition. Recently, ES-242s and several new analogs were isolated from the insect-associated fungus Cordyceps pseudomilitaris BCC 1620.
Atrop isomers of ES-242-4 and -5, (64 and 65, respectively), a C10-C5 dimer (66) and its isomer (67), and two monomers (68 and 69) are new natural products. The C10-C10 dimers (58 – 65), were found to exhibit significant antimalarial activity (P. falciparum K1), with IC₅₀ values of 1.1 – 8.4 µg/mL, but showed much weaker cytotoxic activity (Jaturapat et al., 2001).

A series of xanthone dimers, e.g., ergochrome EE (70), was originally isolated from the sclerotia of Claviceps purpurea (Franck et al., 1966), and later found in fungi in several other non-clavicipitalean families such as Aspergillus spp., Pyrenochaeta terrestris, Penicillium oxalicum, and Phoma terrestris. More commonly, this class of compounds is known as secalonic acids (Cole and Cox, 1981a). The C4-C45 xanthone dimer structures initially presented for secalonic acids (ergochromes) have been correctly revised to C2-C2 dimers (Hooper et al., 1971). Secalonic acid D, the optical antipode of secalonic acid A (70), was shown to be toxic to mice, and secalonic acid A exhibited antimicrobial and phlogistic activities.

Saintopin (71), a reddish purple pigment isolated from Paecilomyces sp., is a novel antitumor antibiotic with topoisomerase II-dependent DNA cleavage (TDC) activity (Yamashita et al., 1990). In the in-vitro TDC activity assay using purified calf thymus topoisomerase II and plasmid DNA, activity of saintopin was detected at 2.5 µM concentration (Yamashita et al., 1991). Saintopin exhibited high in-vitro cytotoxicity against human tumor cell line (HeLa S3 with IC₅₀ of 0.35 µg/ML), and furthermore it showed in-vitro antitumor activity against murine leukemia P388 (ip), exhibiting a statistically significant increase in life span (ILS 30%) at a dose of 25 mg/kg. A water-soluble sulfate analog, UCE1022 (72), was isolated by the same research group from Paecilomyces sp. UCE1022. Compound (72) exhibited inhibitory activity against topoisomerase I, but not topoisomerase II (Fuji et al., 1994). Two other analogs, BM24149-1 (73) and -2 (74), isolated from Paecilomyces sp. BM2419 by a different group (Ishiyama et al., 1998), also acted as topoisomerase I inhibitors.

Hypocrellin A (75), dark red pigment with photodynamic activity toward microorganisms, was isolated from Hypocrella bambusae (Chen et al., 1981). Its analog hypocrellin B (76) was found later from the same species (Zhang et al., 1989).

Naphthalenic anhydride, lamelliconic anhydride (77), its carbonate (78), and several related compounds were isolated from Verticillium lamellicola (McCorkindale et al., 1983). FR-901235 (79), a new immunomodulator produced by Paecilomyces carneus F-4882 (Shibata et al., 1989), has a similar chemical structure to lamelliconic anhydride.

5. NONADRIDES

Nonadrides belong to a compound family that includes formal C₉ units represented as 2-(1-butenyl)-3-methylmaleic anhydrides (Barton and Sutherland,
Although nonadrides have rarely been found from nature, they have been isolated from two species of Clavicipitaceae. Cornexistin (80), isolated from *Paecilomyces varioti* SANK 21086, exhibits herbicidal activity against annual weeds (Nakajima et al., 1991). Compound (81), among the simplest nonadrides from nature, was also isolated from *P. varioti* (Aldridge et al., 1980). Cordyanhydrides A (82) and B (83) from *Cordyceps pseudomilitaris* BCC 1620 (Isaka et al., 2000) are two new nonadrides in which two and three C₉ units are linearly connected. Most of the other known nonadrides consist of two C₉ units connected by head-to-head or head-to-tail coupling to furnish the nine-membered ring. It should also be noted that cordyanhydride B is the first example of a nonadride containing three C₉ units.

6. PEPTIDES

6.1. Cyclosporins

Cyclosporins (or cyclosporines, ciclosporins) are cyclic oligopeptides with 11 amino acid units that are common secondary metabolites produced by fungi of the genus *Tolypocladium*. Several naturally occurring cyclosporins (cyclosporins A–Z) have been characterized to date (Fig. 6) (Ruegger et al., 1976; Traber et al., 1977, 1982, 1987; Jegorov et al., 1995). Among these, cyclosporin A (84) is most well known (Ruegger et al., 1976), and its applications in posttransplantation therapy and treatment of some autoimmune diseases (e.g., psoriasis and rheumatoid arthritis) have greatly drawn scientists’ attention. In addition, the ability of cyclosporins to inhibit T-cell activation has been shown to have a role...
in the treatment of diseases such as nephrotic syndrome, refractory Crohn’s disease and ulcerative colitis, biliary cirrhosis, aplastic anemia, rheumatoid arthritis, myasthenia gravis, and dermatomyositis. However, cyclosporin-derived drugs are known for their toxicity related to kidney problems, increased blood pressure, and tremors.

FIGURE 6  Cyclosporins.
Cyclosporin A exerts its effect by binding to the cytoplasmic cyclophilin A, forming a complex that blocks the phosphatase activity of calcineurin. The cyclophilins are widely distributed and abundant proteins found in eukaryotes and prokaryotes and the cellular receptors for the cyclosporins. In single-crystal form or in chloroform solution, cyclosporin A adopts a distinct backbone conformation with a 9,10-cis peptide bond and three transannular hydrogen bonds. Extensive study has been made concerning the cyclophilin A–cyclosporin A complex structure. Evidence indicates that cyclosporin A adopts an extended conformation. There is no regular secondary structure or intramolecular hydrogen bonds, peptide bonds assume the trans configuration, and polar groups are exposed to the environment. Side chains of residues 1, 4, 6, and 10 are above the macrocyclic ring which engulfs five of the seven NCH₃ groups, while residues 2, 5, 9, and 11 are situated below the plane of the ring (Weber et al., 1991). Cyclosporin A has been proposed to act by inhibiting the cyclophilin-catalyzed cis–trans isomerization of propyl–peptide bonds. However, there is evidence that this is not a requirement for immunological function (Weber et al., 1991). Currently, cyclosporin A and its analogs have been found to possess antiviral activity against AIDS virus (HIV-1) (Billich et al., 1995; Thali, 1995; Sun et al., 1997; Streblow et al., 1998; Briggs et al., 1999).

As for the biosynthesis of cyclosporin A, four head-to-tail acetate units are involved in the formation of the 8-carbon chain of olefinic amino acid, and N-methyl groups of cyclosporin A originate from methionine (Kobel et al., 1983; Zocher et al., 1984). The enzyme named “cyclosporin synthetase” has been shown to be responsible for cyclosporin biosynthesis (Billich and Zocher, 1987; Lawen et al., 1989). This enzyme was subsequently purified and characterized (Lawen and Zocher, 1990).

### 6.2. Linear Peptide Antibiotics

A proline-containing lipohexapeptide, lipohexin (85), was isolated from *Paecilomyces* sp. HKI-0055 and HKI-0096 (Heinze et al., 1997) (Fig. 7). The same research group also obtained this same peptide from *Moeszia lindneri* HKI-0054 (Cylindrocarpon an anamorph of Hypocreaceae). Lipohexin is a competitive inhibitor of propyl endopeptidase (PEP) from human placenta with IC₅₀ of 3.5 mg/mL (Christner et al., 1997). A linear peptide antibiotic, LP237-F8 (86), exhibiting a high level of cytotoxicity and antibacterial activity, was isolated from *Tolypocladium geodes* LP237 (Tsantrizos et al., 1996). This is a new member of the peptibol class of natural products, containing the unusual α-aminoisobutyric and α-amino-α-ethyl-n-pentanoic acids. Another peptibol-like antibiotic, XR586 (87), was isolated and purified from *Acremonium persicinum* X21488 (Sharman et al., 1996).
**FIGURE 7** Peptide antibiotics.
Paecilotoxins (e.g., 88–93), also designated as leucinostatins, isolated from *Paecilomyces lilacinus* (Fukushima et al., 1983a, 1983b), may be the most well known polypeptide mycotoxins from clavicipitalean fungi. Paecilotoxins have strong uncoupling activity against rat liver mitochondria as well as antimicrobial activity. These compounds not only show high cytotoxic activity against various cancer and normal cell lines but also exhibit high oral toxicity (Mikami et al., 1984). *P. lilacinus* is a fungus that has frequently been isolated from various habitats, and systematic studies of paecilotoxin production by strains of *P. lilacinus* from various sources, e.g., soil, insects, and clinical isolates, have been conducted by Mikami et al., (1989). Paecilotoxins were detected from cultures of 19 different isolates among 20 tested. Paecilotoxin A was also isolated from an *Acremonium* sp., an endophyte of European yew (*Taxus baccata*) (Strobe et al., 1997). Finally, the structure proposed for antibiotic P168, isolated from *P. lilacinus* (Isogai et al., 1980; Isogai et al., 1984), was identical to that of paecilotoxin A.

### 7. CEPHALOSPORINS (β-LACTAM ANTIBIOTICS)

Cephalosporins are secondary metabolites produced by fungi of the genus *Cephalosporium, Acremonium, and Penicillium*. Cephalosporins were first discovered in the late 1940s by the Italian scientist, Professor Giuseppe Brotzu, who noticed that cephalosporin C (94) was produced along with penicillin N by the fungus *Cephalosporium acremonium* isolated from sea water near a sewage outlet on the coast of Sardinia. The molecules of cephalosporins possess a β-lactam moiety, similar to that of penicillin antibiotics, and they exhibit potent antibacterial activity against many Gram-positive, Gram-negative, and anaerobic bacteria. It is commonly known that the antibiotics with the β-lactam ring exert their effects by interfering with the structural cross-linking of peptidoglycans in bacterial cell walls (Fountain and Russell, 1969; O’Callaghan et al., 1976; Rolinson, 1980). Among these cephalosporins, the antibiotic cephalosporin C (94) is probably most well known (Abraham and Newton, 1961; Hodgkin and Maslen, 1961), and it has been chemically transformed into several semisynthetic antibiotics. It should be noted that cephalothin was the first commercially available cephalosporin antibiotic, introduced to the market in 1962. Examples of cephalosporin-derived drugs are cefaclor (Ceclor), cefadroxil (Duricef), cefazolin (Ancef, Kefzol, Zolicef), cefixime (Suprax), cefoxitin (Mefoxin), cephalexin (Keflex), and cefuroxime (Ceftin), whose structures are shown in Fig. 8. These drugs are sold in tablet, capsule, liquid, and injectable forms. Although there are a range of side effects of cephalosporin use, their effectiveness outweighs their shortcomings.

The major setback of cephalosporin antibiotics is the development of resistance by bacteria. Such resistance is increasing at an alarming rate and
has become a common problem in primary health care treatment. Several mechanisms of antimicrobial resistance to β-lactam antibiotics have been put forward (McManus, 1997). One important mechanism is the production of β-lactamases, which cleave the β-lactam ring (Bush et al., 1995; Pitout et al., 1998). β-Lactamase activity occurs in both Gram-positive (Staphylococcus aureus and S. epidermidis) and Gram-negative organisms (Haemophilus
influenzae, Neisseria gonorrhoeae, Moraxella [formerly Branhamella] catarrhalis, Escherichia coli, and Proteus, Serratia, Pseudomonas, and Klebsiella species); and also in anaerobic organisms (Bacteroides species). Hence, the use of cephalosporin-derived antibiotics in combination with β-lactamase inhibitors can be highly effective in combating infections caused by β-lactamase-producing organisms.

The amino acids, lysine, α-aminoadipic acid, and cysteine, are precursors in the cephalosporin C biosynthesis (Warren et al., 1967a, 1967b; Huddleston and Abraham, 1978). Study of the incorporation of radioactive-labeled cysteine into cephalosporin C revealed that the formation of its β-lactam ring takes place stereospecifically, with the retention of configuration at C-3 of cysteine (Huddleston and Abraham, 1978). The formation of δ-(1-α-aminoadipyl)-L-cysteinyl-D-valine complex by the enzyme “δ-(1-α-aminoadipyl)-L-cysteinyl-D-valine synthetase” has been found to be the rate-limiting step in the biosynthesis of cephalosporin C (Malmberg and Hu, 1992). Gene recombinant technology and research on genes involved in the biosynthesis of cephalosporins have been employed in order to improve the production of cephalosporins (Diez et al., 1996; Kimura et al., 1996), and several studies on the fermentation technology of cephalosporin production have also been conducted (Zhou et al., 1992a, 1992b, 1993; DeModena et al., 1993; Basak et al., 1995; Kozma and Karaffa, 1996; Karaffa et al., 1999; Araujo et al., 1999; Seidel et al., 2000; Ellaiah et al., 2000; Lee et al., 2001).

8. DIKETOPIPERAZINES

Sulfur-containing diketopiperazine metabolites are produced by a variety of unrelated fungi (Cole and Cox, 1981b), and there have been some examples also from clavicipitalean species (Fig. 9). Verticillins A (95) and B (96), toxins isolated from Verticillium sp. TM-759 (Katagiri et al., 1970; Minato et al., 1973), are dimers of epidithiodiketopiperazines. Verticillin A is cytotoxic to Hela cells with an IC₅₀ value of 0.2 μg/mL; exhibited an antitumor effect against Ehrich asites tumor and showed antiviral activity against polio-virus (Type 1). The acute toxicity (LD₅₀) in mice was found to be 7.6 mg/kg by intraperitoneal administration (delayed toxicity). A related compound, (97), was also isolated from Verticillium tenerum (Hauser et al., 1972). All compounds mentioned are very closely related to chaetocin, which was isolated from Chaetomium minutum (Melanosporaceae) (Hauser et al., 1970), and their monomeric units are related to sporidesmins previously isolated from several species of the genera Sporidesmium and Pithomyces (anamorph, Pleosporaceae). Two bismethylthio diketopiperazines, Sch 54794 (98) and Sch 54796 (99) and an analog, Sch 56396 (100), were isolated from Tolypocladium sp. (Chu et al., 1993, 1997). Related
compounds (101) and (102) were produced by Paecilomyces cf javanica (Rahbæk et al., 1998).

9. CYCLODEPSIPEPTIDES

Beauveria bassiana is a source of various cyclodepsipeptide antibiotics (Fig. 10). Beauvericin (103), the most commonly produced cyclodepsipeptide by strains of B. bassiana (Hamill et al., 1969), exhibits insecticidal activities, i.e., it is toxic to brine shrimp, mosquito larvae, blowfly, and Colorado potato beetle. This compound also exhibits a broad range of antimicrobial activities against Gram-positive bacteria and fungi. There have been reports of isolation of 103 from Paecilomyces fumosoroseus (Bernardini et al., 1975), Polyporus sulphureus (Basidiomycota: Polyporaceae) (Deol et al., 1978), and Fusarium species (anamorph, Hypocreaceae) (Gupta et al., 1991). Later, beauvericin analogs, beauvericins A (104) and B (105), were isolated as minor metabolites from B. bassiana (Gupta et al., 1995) and Paecilomyces tenuipes BCC 1614 (Nilanonta et al., 2000).
Figure 10  Cyclodepsipeptides.
**FIGURE 10**

- **Iserin (117)**
- **Metacyctolin (118)**

- **Aselacin A (119):** $R^1 = OH$, $R^2 = H$, $R^3 = H$
- **Aselacin B (120):** $R^1 = R^2 = O$, $R^3 = OH$
- **Aselacin C (121):** $R^1 = R^2 = O$, $R^3 = H$

- **Destruxin A (122):** $R^1 = CH_2CH=CH_2$
- **Destruxin B (123):** $R^1 = CH_2CH(CH_3)CH_2OH$
- **Destruxin E (124):** $R^1 = CH_2OH$

- **Destruxin A$_1$ (125):** $R^1 = CH_2CH=CH_2$
- **Destruxin Ed$_1$ (126):** $R^1 = CH_2CH(OH)CH_2OH$
- **Destruxin A$_5$ (127):** $R^2 = H$
- **Destruxin A$_6$ (128):** $R^2 = CH_3$
Bassatin (106), a cycloidepsipeptide possessing the same residues as that of cyclohexadepsipeptide (103), was isolated from *B. bassiana* K-717 (Kagamizono et al., 1995). A symmetric cyclooctadepsipeptide displaying insecticidal activity, bassianolide (107), was isolated from *B. bassiana* and *Verticillium lecanii* (Suzuki et al., 1977).

A group of cyclotetradepsipeptides having a 3-hydroxycarboxylic acid residue e.g., (108–116) in Fig. 10, were isolated from *Beauveria* species. There may be confusion, even to those working in the field, due to the three different ways of naming compounds: beauverolides (Elsworth and Grove, 1977, 1980), beauverilide (Isogai et al., 1978), and beauveriolides (Mochizuki et al., 1993; Namatame et al., 1999). Isarolides A–D, isolated from an *Isaria* sp. (Briggs et al., 1968), also belong to the same depsipeptide group. Thus, the structure of beauverolide B₄ (116), an isomer of beauverolide B, bearing D-allo-isoleucine residue (instead of D-isolucine in B), is identical with beauverilide A and isarolide A. The structure proposed for beauverolide L (Jegorov et al., 1994) is identical with that presented for beauveriolide II (113). Depsipeptide SCH 58149, isolated from *Acremonium* sp. (Hedge et al., 1998), has an identical planar structure to beauveriolide I (112), although it should be noted that the stereochemical integrity of SCH 58149 was not addressed in the report.

*Isaria* species are the source of isarin (117) and its three analogs, isariins B, C, and D, cyclohexadepsipeptides having a 3-hydroxydodecanoic acid or 3-hydroxynonanoic acid residue (Vining and Taber, 1962; Deffieux et al., 1981). The structure of an immunosuppressive substance, metacytofilin (118), from *Metarhizium* sp. TA2759, with an unusual chemical skeleton was determined by X-ray crystallography (Iijima et al., 1992). Aselacins A–C (119–121), novel cyclopentadepsipeptides attached to a functionalized long-chain fatty acid, were isolated from *Acremonium* spp. AB 2093T-194 and AB 2086L-51 (Hochlowski et al., 1994). Aselacin A inhibits the binding of endotherin-1 to its receptor with IC₅₀ of 20 mg/mL (Jackson et al., 1994).

Destruxins (e.g., 122–128) are a group of cyclohexadepsipeptides exhibiting various biological activities, such as insecticidal, phytotoxic, antiviral, cytotoxic, and immunodepressance. After the report of isolation of the first destruxins in 1961, e.g., A (122), C (123), and E (124), from *Metharizium anisopliae* (Kodaira, 1961), more than 30 analogs have since been found from this same fungus (Jegorov et al., 1998). This class of compounds have also been isolated from another entomopathogenic fungus, *Aschersonia* sp. (Krasnoff et al., 1996), and plant-pathogenic fungi, *Alternaria brassicae* (anamorph, Pleosporaceae) and *Trichothecium roseum*. 

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10. ALKALOIDS

10.1. Ergot Alkaloids

It is known that the genus *Claviceps* not only causes a disease to cereal crops and grasses, which reduces yield and quality of grains and hay, but it is also harmful to livestock fed infected grains or hay. The disease, generally known as “ergotism” was first described in the 1800s, although the connection between ergot fungus and epidemics among humans and livestock was known several hundred years earlier. Human poisoning was common in Europe in the Middle Ages, when toxic amounts of ergotted rye bread was consumed. The disease caused by *Claviceps* is generally more prevalent in rye, but significant losses have also been reported in spring wheat, durum, barley, and other small grains. Although the crop loss caused by this disease is of economic importance, the effect of ergot’s alkaloid toxins on humans and animals is of much greater significance.

Ergot alkaloids are secondary metabolites produced by *Claviceps purpurea*, *C. paspali*, *C. fusiformis*, and species of *Balansia* (Bacon et al., 1979). They possess a tetracyclic ring system commonly known as ergoline (Fig. 11) as a common part of their molecules, Ergot alkaloids can also be produced by fungi of other families such as *Penicillium* (Padmanabha et al., 1998) and *Aspergillus* (Janardhanan et al., 1984). Generally, ergot alkaloids are divided into four main structural groups, clavines or elymoclavines (A), lysergic acids (B), lysergic acid amides or ergometrines (C), and ergopeptines or

![Figure 11](image_url)  
**FIGURE 11** Examples of ergot alkaloids.
ergotamines (peptide alkaloids) (D) (Betina, 1994) (Fig. 11). To date, several naturally occurring alkaloids in this class have been isolated, and they are extensively reviewed elsewhere (Flieger et al., 1997; Mukherjee and Menge, 2000). Among a number of biological activities, ergot alkaloids induce direct stimulation of smooth muscle, particularly in their ability to intensify uterine contractions, and selective blocking of the sympathetic nervous system. Despite the fact that lysergic acid diethylamide (LSD) (Fig. 12), the most notorious and controversial ergot alkaloid possessing hallucinogenic properties, is sometimes involved in drug abuse, extensive research on medical applications have been performed which has led to the discovery of drugs for the treatment of migraine, inhibition of lactation, and Parkinson’s disease. Dihydroergotamine and methysergide are examples of commercially available drugs for migraine. Bromocryptine is commercially available for prolactin inhibition. Other unique biological activities of ergot alkaloids, especially for the treatment of Parkinson’s disease, are still of great interest (de Groot et al., 1998; Blanchet, 1999; Sit, 2000).

Biosynthesis of ergot alkaloids has received wide attention and extensive studies have been made (Riederer et al., 1996; Walzel et al., 1997). This has led to the identification of several enzymes involved in the biogenetic pathway.
Apart from these, production of ergot alkaloids by fermentation technology has been widely studied (Banks et al., 1974; Rehacek and Kozova, 1975; Rehacek et al., 1977; Robbers, 1984; Kren et al., 1984).

**FIGURE 13** Alkaloids.

(Shibuya et al., 1990; Tsai et al., 1995; Tudzynski et al., 1999).
10.2. Other Nitrogen-Containing Compounds from Clavicipitalean Fungi

Balanol (129), produced by *Verticillium balanoides*, is a novel and highly potent inhibitor of protein kinase C (PKC) (Kulanthaivel et al., 1993) (Fig. 13). IC\textsubscript{50} values of 4–9 nM were reported in assays against human PKC enzymes \(\alpha\), \(\beta-I\), \(\beta-II\), \(\gamma\), \(\delta\), \(\epsilon\), and \(\eta\), with the exception of \(\zeta\) (150 nM). Extensive work has been conducted by many research groups involving the synthesis of balanol analogs in order to find more potent and selective PKC inhibitors (Koide et al., 1995; Lai et al., 1997). The structure proposed for ophiocordin (130), an antifungal antibiotic isolated from *Cordyceps ophioglossoides* (König et al., 1980), was regioisomeric to balanol. It was also clarified later that *V. balanoides* and *C. ophioglossoides* produce an identical compound, the structure being that assigned to balanol (Boros et al., 1994).

Two novel compounds, UCS1025A (131) and B (132), were isolated from *Acremonium* sp. KY4917. UCS1025A exhibits antiproliferative activity against human tumor cell lines, with IC\textsubscript{50} values ranging from 21 nM to 58 \(\mu\)M, and it also shows moderate antimicrobial activity against Gram-positive bacteria (Nakai et al., 2000). Structurally, however, the stereochemistry of UCS1025 s was not presented in the report.

The highly functionalized pyridones, yellow pigments, tenellin (133) and bassianin (134), were isolated (El Basyouni et al., 1968) and identified (Wat et al., 1977) from *Beauveria tenella* and *B. bassiana*, respectively. Identities of pyridovericin (135) and an analog bearing a macrolide subunit, pyridomacrolidin...
Pyridoxatin (137), from Acremonium sp. BX86, is a free-radical scavenger. This compound inhibits lipid peroxidation induced by free radicals in rat liver microsomes with IC$_{50}$ of 0.55 µg/mL (Teshima et al., 1991). The structure of tolypocin [HL], isolated from Tolypocladium geodes (Jegorov et al., 1993), is believed to be identical to that of pyridoxatin, although absolute configuration of the latter has not been determined. Cordypridine A (138) and its atrop isomer, cordypridine B (139), isolated from Cordyceps nipponica BCC 1389, exhibits potent antimalarial activity against Plasmodium falciparum (K1, multi-drug-resistant strain) with IC$_{50}$ values of 66 and 37 ng/mL, respectively, while their cytotoxicity was much weaker (Isaka et al., 2001b). Related tricyclic analog (140 and 141) were also isolated from the same fungal strain (BCC 1389).

Paspaline (142), paspalicine (143), paspalinine (144), and pasparitrem A (145) and B (146), isolated from Claviceps paspali (Fehr and Acklin, 1966), belong to the paspalitrem group of the tremorgen. Compounds (144), (145), and (146) have been strongly implicated in the “Paspalum stagger” syndrome (Gieger and Barrentine, 1939). It has been shown that these tremorgenic compounds are present in C. paspali sclerotia from toxic pastures (Cole et al., 1977).

11. MISCELLANEOUS SECONDARY METABOLITES

Several sphingoid derivatives have been isolated: sphingofungins E (147) and F (148) from Paecilomyces variotii ATCC 74097 (Horn et al., 1992), ISI-I (149) from Isaria sinclairii (Fujita et al., 1994), and compounds (150) and (151) from Epichloë typhina (Koshino et al., 1992b) (Fig. 14). Sphingofungins E and F exhibited serinepalmitoyltransferase inhibitory activity as known for previously reported sphingofungins. The antifungal ISI-I is identical with myriocin and thermozymocidin previously isolated from Myrothecium albobrunum and Mycelia sterilia, respectively. ISI-I exhibited immunosuppressive activity 10–100 times more potent than cyclosporin A. Since (149) inhibited the proliferation of an IL-2-dependent mouse cytotoxic T-cell line, CTLL-2, but not the production of IL-2, unlike cyclosporin A and FK-506, extensive synthetic studies of this compound and its analogs have been done by several groups (Fujita et al., 1996).

Cordycepin (3\textsuperscript{-}deoxyadenosine; 152), isolated from Cordyceps militaris (Cunningham et al., 1951; Bentley et al., 1951), is a deoxynucleoside possessing antifungal, antiviral, and antitumor activity. Although (152) was isolated 50 years ago, there are still an increasing number of publications on the biochemistry of this compound and other nucleoside mimics. It should be noted that an adenosine derivative, N\textsuperscript{\textcircled{-}}- (2-hydroxyethyl) adenosine (153), was isolated, together with (152) and adenosine, from several species of Cordyceps and Isaria (Furuya et al., 1983).
Among Clavicipitalean fungi, *Cordyceps sinensis* is most well known and has long been used in Chinese traditional medicine. The fruiting bodies of *C. sinensis*, which produce the active ingredients, have a rich and interesting history. For generations, *C. sinensis* has been considered as the premier herbal medicine in the Chinese culture for restoring energy, promoting longevity, 

**Figure 14** Miscellaneous metabolites of Clavicipitaceae.

**12. CLAVICIPITALEAN FUNGI IN TRADITIONAL MEDICINE**

Among Clavicipitalean fungi, *Cordyceps sinensis* is most well known and has long been used in Chinese traditional medicine. The fruiting bodies of *C. sinensis*, which produce the active ingredients, have a rich and interesting history. For generations, *C. sinensis* has been considered as the premier herbal medicine in the Chinese culture for restoring energy, promoting longevity,
and improving the quality of life. This fungal material is extremely rare; it is normally found at altitudes above 4000 m in the highlands of Southern China, Tibet, and the Eastern Himalayas. It is said it takes 5–7 years for fruit bodies of *C. sinensis* to complete their life cycle and produce the essential natural products. In the past, the use of *C. sinensis* was reserved exclusively for the Emperor’s Palace, due to its scarcity and extremely high price.

In traditional Chinese medicine, *C. sinensis* is prepared as a water extract or cooked. The tonic has been consumed safely for hundreds of years by local people, who referred to it as “Dong-Zhong-Chang-Cao,” which translates as “winter worm, summer grass” (sometimes it is referred to as “Dong-Gong Xia Cao,” which means winter insect, summer grass).

Recently, scientists have paid great attentions to the fungus *C. sinensis*, and several studies on its biological activities have been carried out. An extract of *C. sinensis* exhibited antioxidation activity (Yamaguchi et al., 2000a; Li et al., 2001), immunomodulatory activity (Kuo et al., 1996, 2001; Chiu et al., 1998), hypoglycemic activity (Kiho et al., 1999), hypotensive and vasorelaxant activities (Chiou et al., 2000), and antitumor activity (Chen et al., 1997; Bok et al., 1999). The fungus *C. sinensis* also proved beneficial in the prevention of atherosclerotic lesions induced by oxidative stress (Yamaguchi et al., 2000b) and inhibition of proliferation of cultured human glomerular mesangial cells induced by LDL (Zhao-Long et al., 2000). Scientific evidence of the previous ancient Chinese herbal regimen of *C. sinensis* was extensively reviewed by Zhu et al. (1998a, 1998b). Although *C. sinensis* is widely commercially available in the form of tonic, crude extract, formulated herbal medicine, and its fruiting bodies, only a few biologically active substances, which include polysaccharides (Kiho et al., 1986) and sterols (Bok et al., 1999), have been chemically characterized up until today. It seems that the clarification of the active principle(s) of *C. sinensis* will remain research topics in this new millennium.

13. SUMMARY

As reviewed in this chapter, the family Clavicipitaceae is a rich source of biologically active secondary metabolites. Important discoveries from this family are summarized in Table 1. Certain genera such as *Acremonium*, *Verticillium*, *Paecilomyces*, *Beauveria* and *Tolypocladium* seem to be endless sources of novel compounds. In contrast, the number of new compounds reported so far is relatively small for the insect host-specific groups, such as *Cordyceps*, *Aschersonia*, *Hypocrella*, *Akanthomyces*, and *Torrubiella*. It is believed that this is not due to the poor diversity of these fungi or their metabolites but because of the unavailability of these fungi for novel metabolite studies. At the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand, Dr. Nigel L. Hywel-Jones’ group has been systematically collecting
<table>
<thead>
<tr>
<th>Producing fungus (original)</th>
<th>Compound</th>
<th>Chemical class</th>
<th>Bioactivity</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cephalosporium acremonium</em></td>
<td>Cephalosporin C (94)</td>
<td>β-Lactam</td>
<td>Antibacterial</td>
<td>1948</td>
</tr>
<tr>
<td><em>Tolypocladium polysporum</em></td>
<td>Cyclosporin A (84)</td>
<td>Oligopeptide</td>
<td>Immunosuppressant</td>
<td>1976</td>
</tr>
<tr>
<td><em>Claviceps purpurea</em></td>
<td>Ergot alkaloids</td>
<td>Alkaloid</td>
<td>Dopamine receptor antagonist</td>
<td>1918</td>
</tr>
<tr>
<td><em>Cordyceps sinensis</em></td>
<td>???</td>
<td>???</td>
<td>Eternal youth</td>
<td>BC</td>
</tr>
<tr>
<td><em>Claviceps paspary</em></td>
<td>Pasparitrem (114, 115, 116)</td>
<td>Alkaloid</td>
<td>Tremorgen</td>
<td>1966</td>
</tr>
<tr>
<td><em>Cordyceps militaris</em></td>
<td>Cordycepin (152)</td>
<td>Deoxynucleoside</td>
<td>Antivirus, antitumor</td>
<td>1951</td>
</tr>
<tr>
<td><em>Cordyceps nipponica</em></td>
<td>Cordypyridones (138, 139)</td>
<td>Alkaloid</td>
<td>Antimalarial</td>
<td>2001</td>
</tr>
<tr>
<td><em>Paecilomyces inflatus</em></td>
<td>Sesterterpene (8)</td>
<td>Sesterterpene</td>
<td>GPI inhibitor</td>
<td>1998</td>
</tr>
<tr>
<td><em>Paecilomyces lilacinus</em></td>
<td>Paecilotoxin A (88)</td>
<td>Oligopeptide</td>
<td>Mycotoxin, antitumor</td>
<td>1983</td>
</tr>
<tr>
<td><em>Verticillum balanoides</em></td>
<td>Balanol (129)</td>
<td>Alkaloid</td>
<td>PKC inhibitor</td>
<td>1993</td>
</tr>
</tbody>
</table>
insect-pathogenic fungi over the last decade from various parts of Thailand. More than half of the isolates, > 1000, cannot be assigned to any previously named species. Systematic biological activity-guided isolation of these fungi (deposited at the BIOTEC Culture Collection, BCC) has led to the identification of several new compounds having antimalarial or antimycobacterial activities, such as (10, 46, 47, 64-69, 82, 83, 138–141). Thus, it is very clear that this group of fungi is still not thoroughly explored, and there remains significant potential for novel discovery.

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13

Molecular Genetics of Ergot Alkaloid Biosynthesis

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1. INTRODUCTION

Ergot alkaloids are metabolites, produced by various members of the Clavicipitaceae, that contain the characteristic four-membered ergoline ring and that, generally, may be considered as precursors to, or derivatives of, lysergic acid. These include clavine alkaloids, simple amides of lysergic acid, and ergopeptines. Different ergot alkaloid-producing fungi produce different subsets of alkaloids among the complex group observed collectively. The ergot alkaloids best known for toxicity are the ergopeptines (e.g., ergotamine and ergovaline), in which lysergic acid is incorporated into nonribosomally synthesized peptides and, to perhaps a lesser extent, simple amides of lysergic acid (Rutschman and Stadler, 1978; Waller and Dermer, 1981).

Numerous clavicipitaceous fungi, including some members of the genera Claviceps, Epichloë (and their closely related Neotyphodium anamorphs), and Balansia, produce ergot alkaloids. These fungi typically occur as pathogens or
mutualistic endophytes of grasses. The ergot alkaloids they produce are hypothesized to play roles as antiherbivore compounds and may have other, heretofore unrecognized, roles in the plant–fungus–herbivore interactions in which they are found. However, because ergot alkaloid-producing fungi typically produce additional classes of bioactive metabolites, a clear role for ergot alkaloids has been difficult to assign. A molecular genetic approach in which specific ergot alkaloid biosynthesis genes can be inactivated (e.g., knocked out) provides a strategy to address the significance of ergot alkaloids to their producing fungi and the interactions in which they are found. The ability to manipulate ergot alkaloid biosynthesis may have practical applications in agriculture (where ergot alkaloids cause toxicoses in grazing animals) and medicine (where ergot alkaloids and derivatives are used in a variety of clinical applications).

In this chapter, our objectives are to: summarize the biochemical pathway by which ergot alkaloids are synthesized; discuss the agricultural importance of ergot alkaloid-producing fungi and the toxins they produce; describe the biochemistry and molecular biology of two ergot alkaloid biosynthesis genes that have been cloned recently; and, summarize ongoing work to manipulate these genes to eliminate ergopeptines or all ergot alkaloids for basic and applied studies.

2. BIOCHEMICAL PATHWAY FOR ERGOT ALKALOIDS

2.1. Synthesis of the Ergoline Multiring Nucleus

Many of the intermediates and several of the enzymatic steps involved in the biosynthesis of ergot alkaloids have been studied in Claviceps species (e.g., Floss, 1976; Waller and Dermer, 1981; Kozikowski et al., 1988; Shibuya et al., 1990) and are outlined in Figs. 1–3. However, much about the pathway remains to be investigated. A similar pathway is expected in Neotyphodium, Epichloë, and Balansia spp. given their phylogenetic relationship to Claviceps (Glenn et al., 1996; Craven et al., 2001) and the presence of similar DNA sequences for two of the genes in the pathway (described below).

The early steps in the ergot alkaloid biosynthetic pathway are outlined in Fig. 1. The first determinant and rate-limiting step is the prenylation of tryptophan to 4-(\(\gamma,\gamma\)-dimethylallyl)tryptophan (DMAT), catalyzed by dimethylallyl-diphosphate:L-tryptophan dimethylallyltransferase (DMAT synthase; EC:2.5.1.34) (Heinstein et al., 1971; Gebler and Poulter, 1992). The prenyl group for the DMAT synthase reaction is provided in the form of dimethylallyl diphosphate (DMAPP), which is derived from mevalonic acid. After the formation of DMAT, the free amino group of this intermediate is N-methylated with a methyl group donated by \(S\)-adenosylmethionine (AdoMet). The N-methylated DMAT is then converted into chanoclavine I by closure of the
third, or “C,” ring of what will ultimately be the four-membered ergoline ring structure. (Rings A and B are the indole rings from tryptophan.) Formation of chanoclavine I from DMAT requires a series of reactions perhaps involving cytochrome P450 and tertiary alcohol, diene, and epoxide intermediates of the side chain originally derived from DMAPP (Kozikowski et al., 1988). Chanoclavine I is the entry point into the clavine family of ergot alkaloids, as the fourth, or “D,” ring in this molecule is cyclized via an aldehyde intermediate into agroclavine in a reaction catalyzed by chanoclavine I cyclase (Erge et al., 1973, as reviewed in Floss, 1976). Agroclavine may be oxidized into elymoclavine, a precursor to lysergic acid. Numerous other clavines, differing in the presence or position of the double bond in the D ring and/or the degree of oxidation of the D ring or its side chain, have been isolated from various ergot alkaloid-producing fungi (Floss, 1976; Waller and Dermer, 1981). Among the clavines, only elymoclavine may be converted into lysergic acid by isomerization of the double bond in the D ring and oxidation of its side chain. Lysergic acid has been difficult to detect in ergot alkaloid-producing fungi and accumulates only in various amide and peptide forms (Floss, 1976; Waller and Dermer, 1981).

2.2. Simple Amides of Lysergic Acid

Lysergic acid-derived ergot alkaloids include three simple amides—lysergic acid amide (LSA or ergine), lysergic acid-2-hydroxyethylamide (lysergyl-methylcarbinolamide) and ergonovine (2-propanolamide side chain) (Fig. 2)—as well as ergopeptines (Fig. 3). The important question of the biosynthesis of the simple amides has not been addressed in the literature recently. Early feeding studies indicate that only labeled alanine, among several potential precursors tested, could serve as the precursor of the 2-hydroxyethyl amide and 2-propanolamide side chains, and that lysergic acid amide is not a precursor to the two other amides (reviewed in Floss, 1976). If, as these findings indicate, the simple amides of lysergic acid are derived from a lysergyl-alanine intermediate, then the formation of such an intermediate may involve a peptide synthetase similar to the lysergyl peptide synthetase described below.

2.3. Ergopeptines

Ergopeptines are synthesized from D-lysergic acid and three L-amino acids via a thiotemplate mechanism similar to that responsible for the biosynthesis of numerous microbial peptide toxins and antibiotics (reviewed in Marahiel et al., 1997; von Döhren et al., 1997). An enzyme system with peptide synthetase activities for D-lysergic acid and the three L-amino acids of ergotamine has been isolated from C. purpurea and designated lysergyl peptide synthetase (LPS) (Riederer et al., 1996; Walzel et al., 1997). The product of LPS is a lysergyl-tripeptide lactam (Fig. 3). The α carbon of the amino acid attached to lysergic
DMAPP + Trp \rightarrow \text{DMAT synthase} \rightarrow \text{DMAT}

CH₃ from AdoMet

Chanoclavine cyclase

Clavines

R=CH₃=agroclavine
R=CH₂OH=elymoclavine

ergopeptines

simple amides of lysergic acid

Lysergic acid
acid (L-alanine in the case of ergotamine or ergovaline; marked with an asterisk in Fig. 3) is directly hydroxylated via molecular oxygen (Quigley and Floss, 1980). The hydroxylated intermediate is proposed to spontaneously cyclize to the final cyclo ring characteristic of ergopeptines (Fig. 3) (Floss, 1976; Quigley and Floss, 1980).

Numerous ergopeptines have been isolated that have the same general structure but with substitutions at the amino acid positions in the tripeptide

**Figure 2** Simple amides of lysergic acid.

**Figure 1** Key intermediate and enzymes from the first committed step in ergot alkaloid biosynthesis through the synthesis of lysergic acid. Simple amides of lysergic acid and ergopeptines are covered in Figs 2 and 3, respectively. Abbreviations: DMAPP = dimethylallyl pyrophosphate; DMAT = dimethylallyl tryptophan; AdoMet = S-adenosyl-methionine.
Biosynthesis of ergopeptines from lysergic acid. Ergovaline is presented as an example. Various other ergopeptines differ at amino acid positions I and II as indicated in the table, or, in one case, in position III as indicated in the text. The asterisk in the diagram of lysergyl-alanyl-valyl-proline lactam indicates the position of the carbon that is hydroxylated prior to cyclol ring formation.
portion of the molecule. All 12 ergopeptines conceivable by substitutions of three L-amino acids at position I and four L-amino acids at position II have been characterized (Fig. 3). An additional noteworthy ergopeptide not listed in Fig. 3 is the nonprolyl peptide, ergobalansine. Ergobalansine contains L-alanine at position III rather than L-proline; positions I and II contain L-alanine and L-valine, respectively, similar to ergovaline. Ergobalansine was originally isolated from Balansia spp. (Powell et al., 1990), and also has been detected in the green plant Ipomoea piurensis (Jenett-Siems et al., 1994). All ergopeptines also are found as their physiologically inactive “-inine” epimers (e.g., ergovalinine as an epimer of ergovaline), which result from epimerization of the bond between the carbon of the amide linkage and the ergoline ring. It is likely that epimerization occurs during extraction, so it is difficult to discern if these epimers are also natural metabolites.

3. AGRICULTURAL SIGNIFICANCE AND ECOLOGICAL ROLES OF ERGOT ALKALOIDS

3.1. Role of Claviceps purpurea

Ergot alkaloid-producing fungi in the genera Claviceps and Neotyphodium have had a particularly strong impact on agriculture because they produce ergopeptines in association with plants that are food sources for people and/or livestock. Within the genus Claviceps are ovarian pathogens of rye, wheat, sorghum, and other wild and cultivated grasses. These fungi colonize the pistils of their host plants and replace the developing seed with their own sclerotia (ergots). The sclerotia, often containing large quantities of ergot alkaloids, may then be harvested with the grain and ingested by people or livestock. Symptoms associated with ergot poisoning include increased blood pressure and body temperature, destruction of the nervous system, reduced reproductive capacity and lactation, and gangrene of the extremities (Berde and Stürmer, 1978; Clark et al., 1978).

Because ergot poisoning has been fairly common in human history, and because of the combination of hallucinogenic and overt toxicological effects, several major historical events have been hypothesized to involve, at least as a contributing factor, episodes of ergotism. Examples include an outbreak that thwarted a planned invasion of the Ottoman Empire by Peter the Great of Russia (Kavaler, 1965), the Salem Witch Trials in colonial Massachusetts (Matossian, 1989), the “Great Fear” preceding the French Revolution (Matossian, 1989), and the Crusades (Billings, 1996). More recently, the exploitation of the ergot alkaloid derivative D-lysergic acid diethylamide (LSD) as a popular hallucinogen has brought renewed attention to the effects of ergot alkaloids.
3.2. Role of Ergot Alkaloid-Producing Endophytes

Neotyphodium spp. are anamorphic relatives of the Clavicipitaceae that grow endophytically in many grass species. Of particular agricultural importance is the symbiosis of *N. coenophialum* with the common pasture grass, tall fescue (*Festuca arundinacea*). Unlike *Claviceps* spp., which produce ergot alkaloids only in developing seed heads, *N. coenophialum* produces ergot alkaloids throughout the growing season and in various tissues of the plant, including leaf sheaths, leaves, and seeds (Lyons et al., 1986; Rottinghaus et al., 1991). *Neotyphodium coenophialum* is not pathogenic, but rather lives symbiotically in above-ground parts of the plant including developing seeds. Mycelium of the fungus survives in the seed and is transmitted vertically to the next-generation seedling. This endophyte is responsible for numerous fitness enhancements that help make tall fescue one of the most widely used plants for forage, soil conservation, and land reclamation in the United States (Ball et al., 1993; Schardl and Phillips, 1997). However, animals grazing *N. coenophialum*-infected tall fescue ingest significant amounts of ergot alkaloids, in particular ergovaline. Because *Neotyphodium* species are systemic in the plant and concentrated near the growing points (ca. soil level), the associated ergot alkaloid toxicity problem is most severe with heavy grazing. Moreover, when animals are under temperature stress, the endophyte can cause widespread toxicosis problems (Raisbeck et al., 1991). Symptoms associated with livestock grazing endophyte-infected fescue include reduced weight gain, hormonal imbalances leading to reduced fertility and lactation, and, in exceptional cases, gangrene of the animal’s limbs (Putnam et al., 1991; Chestnut et al., 1992; Peters et al., 1992; Porter and Thompson, 1992). Nearly two decades of research by several groups worldwide has provided evidence that the ergot alkaloids are the main toxic factors in *N. coenophialum*-associated “tall fescue toxicosis” (Bacon and White, 1994). Experiments involving the feeding or injection of purified or synthesized ergopeptines indicate that at least some of the observed symptoms of animal poisoning by feeding on grasses infected with ergot alkaloid producers are indeed attributable to the ergopeptines (Zhang et al., 1994; Spiers et al., 1995). Still, the contributions of other known and unknown metabolites to animal toxicity have not been determined. Therefore, a direct genetic test of the importance of ergovaline and other ergot alkaloids in animal toxicosis would be highly informative.

Perennial ryegrass (*Lolium perenne*) forms similar associations with *Neotyphodium lolii* or, more rarely, a second species of *Neotyphodium* classified as LpTG-2 [for *L. perenne* endophyte taxonomic grouping 2 (Christensen et al., 1993)] and exemplified by the isolate Lp1. LpTG-2 is a hybrid of *N. lolii* × *Epichloë typhina* (Schardl et al., 1994), and plants with this hybrid differ in their alkaloid profiles from those with *N. lolii* (Christensen et al., 1993; Bony et al.,...
Both of these perennial ryegrass endophytes produce ergovaline, but *N. lolii* also produces lolitrems, indole-diterpene alkaloids believed to be responsible for the ryegrass staggers syndrome. Because of its much lower lolitrems production, Lp1 was initially included in perennial ryegrass cultivars marketed in New Zealand, but was withdrawn in 1992 due to its high-level production of the ergopeptine ergovaline (Anonymous, 1992).

Whereas the agricultural significance of these compounds is considered to be great, the true ecological roles of ergot alkaloids remain elusive. Although the ergot alkaloids have varied and potent biological activities, the linkage of these activities to benefits of the producing organisms is difficult to make. No antimicrobial activities are reported for these compounds, which instead act on neurotransmitter receptors and reuptake transporters. Moreover, the secondary metabolic complexity of ergot alkaloid-producing fungi and related non-producers has confounded investigation of the roles of ergot alkaloids. Fungi that produce ergot alkaloids also typically produce at least one other type of bioactive alkaloid, such as the 1-aminopyrrolizidines (lolines) (Blankenship et al., 2001), pyrrolopyrazines (peramine) (Rowan and Gaynor, 1986), and indole-diterpene tremorgens (lolitrems) (Esser and Tudzynski, 1978; Dorner et al., 1984; Rowan, 1993). [Interestingly, tremorgens, such as lolitrems and paspalitrems, are produced from the same precursors as clavines and ergot alkaloids, but have very different structures and biological activities (Knaus et al., 1994; Munday-Finch et al., 1997; Young et al., 2001).] Because of these differences in other classes of bioactive alkaloids among ergot alkaloid-producing fungi and related non-producers, the roles of ergot alkaloids in vivo have been difficult to assess. However, with the capability to transform ergot alkaloid-producing fungi (Murray et al., 1992; Tsai et al., 1992) and to specifically knock out genes for steps in the biosynthetic pathway (Panaccione et al., 2001), more definitive tests may soon be conducted.

It is difficult to conceive of the antimammalian symptoms specifically representing significant fitness advantages to fungi that produce ergot alkaloids. Perhaps the most important benefit to the fungus is reduction in the appetite of the animal. Thus, animals that proceed to graze a *Claviceps*- or endophyte-infected stand will consume considerably less of the biomass—both plant and fungus—than when ergot alkaloids are absent. Where the fungus may represent a very large clone, as could be the case for the endophytes, it is conceivable that the clone might exhibit an increased survivability. However, this possibility has not been tested or modeled to determine if ergot alkaloid synthesis provides any significant evolutionary advantage to the fungus in the context of grazing vertebrates.

Another possible advantage is conferred by deterrence of insect predation on the fungus or the plant that represents its food source. Ergopeptines in an artificial diet have been shown to deter feeding of adult beetles (*Heteronychus arator*)
from the scarab family (Ball et al., 1997), and when supplied at a relatively high concentration, ergopeptines deter larvae of fall armyworm (*Spodoptera frugiperda*) (Clay and Cheplick, 1989). The hairy chinch bug is also negatively affected by ergovaline-producing endophytes (Saha et al., 1987; Mathias et al., 1990; Carrière et al., 1998; Richmond and Shetlar, 2000). Nevertheless, other metabolites produced alongside ergot alkaloids tend to be more potent insecticides or feeding deterrents. For example, the lolines have broad-spectrum insecticidal activity and, at physiological concentrations, kill or deter feeding by important plant pests such as several aphid species (Riedell et al., 1991; Wilkinson et al., 2000). Similarly, peramine deters feeding of some insect species at field concentrations, and, for example, seems to be an essential bioprotective agent against Argentine stem weevil in New Zealand (Rowan, 1993; Rowan and Gaynor, 1986). Indolediterpenes produced by *Claviceps*, *Epichloë*, and *Neotyphodium* species, among others, also tend to exhibit anti-insect activities (Rowan, 1993). An untested possibility is that ergot alkaloids may act synergistically with these other metabolites in protecting the fungus or infected plant.

4. **REGULATION OF ERGOT ALKALOID EXPRESSION**

4.1. **Regulation In *Claviceps purpurea***

In keeping with other secondary biosynthesis in microorganism, ergot alkaloid biosynthesis in *C. purpurea* occurs at a specified stage in the life cycle. In particular, synthesis is associated with the onset of sclerotium (ergot) differentiation (Didek-Brumec et al., 1996). Considerable efforts have been made to promote fermentative production of ergotamine for the pharmaceutical industry. Early observations (Spalla, 1973) suggested that some ergotamine-producing strains are heterokaryotic. When certain single-spore isolates were grown together, a “giant,” fast-growing, fluffy colony phenotype emerged, and such giant colonies were capable of producing ergot alkaloid even though the original single-spore isolates were not. However, it is doubtful that most ergot alkaloid-producing cultures are heterokaryotic. Still, Spalla’s observation is intriguing in light of remaining questions of the sexual life cycle of *C. purpurea*. Esser and Tudzynski (1978) report that this fungus is homothallic (self-compatible). Generally, *Claviceps* perithecia form directly upon germination of the sclerotium, without prior mating to another strain. Therefore, these sclerotia must contain the necessary genetic composition for meiosis. Since many other Clavicipitaceae are heterothallic, requiring fertilization of one mating type by the other (White and Bultman, 1987; White and Owens, 1992; White et al., 1995), it is possible that *C. purpurea* can grow as a diploid, aneuploid or stable heterokaryon, with genetic determinants of both mating types. Such diploid or heterokaryotic strains would not require fertilization to enter the sexual life cycle.
Interestingly, the two *C. purpurea* strains so far investigated possess two or more copies each of the ergot alkaloid biosynthesis genes encoding DMAT synthase (Wang, 2000) and lysergyl peptide synthetase (Wang, 2000, Panaccione et al., 2001) (both of these genes described below), suggesting that they may have evolved from a diploid or polyploid state. This, then, may account for their abilities to self-mate, and to produce presclerotial cell types and ergot alkaloids in submerged culture.

Ergot alkaloid production in fermentation cultures is tightly regulated (Krupinski et al., 1976). Increasing phosphate levels from 5 to 22 mM suppresses production. In contrast, extensive culture studies with tryptophan and tryptophan analogs indicate that ergotamine production is substrate-inducible. Furthermore, tryptophan and phosphate regulation is associated with increases in DMAT synthase activity, suggesting that this determinant step is also the rate-limiting step for the pathway. Expression of DMAT synthase is regulated at least in part at the level of its mRNA (Arntz and Tudzynski, 1997).

### 4.2. Regulation in *Neotyphodium coenophialum*

The plant environment is somehow conducive for ergot alkaloid biosynthesis by *N. coenophialum*. When this fungus is grown on complex medium, ergovaline is not generally produced. Even the minimal medium that induces loline production by *Neotyphodium uncinatum* (Blankenship, 2001) fails to induce ergovaline (or loline) production by *N. coenophialum* (J. Wang, M. R. Siegel, W. Hollin, and C. L. Scharl, unpublished data). A complex and lengthy culture procedure described by Bacon (1988) results in ergovaline production of some isolates, apparently when the fungus is in stationary phase. Nevertheless, in *symbio* production of ergovaline is highly reliable, at least for *N. coenophialum* occurring naturally in tall fescue cv. Kentucky 31 and its derivatives. The ergot alkaloid biosynthesis gene *dnaW* (encoding DMAT synthase) was transcribed in *symbio* but not in cultured *N. coenophialum*, indicating that ergovaline production also is regulated, at least partly, at the level of mRNA expression (Wang, 2000).

### 5. GENES ENCODING ENZYMES INVOLVED IN ERGOT ALKALOID BIOSYNTHESIS

#### 5.1. DMAT Synthase

##### 5.1.1. Biochemistry

Shibuya et al. (1990) and Gebler and Poulter (1992) provided experimental support that DMAT synthase catalyzes an electrophilic aromatic substitution (Fig. 4) similar to that of other prenyl transferases such as farnesyl diphosphate synthase. A positively charged alkyl intermediate—an allyl carbocation—is
thought to be generated upon hydrolytic removal of the diphosphate moiety. Then the allyl carbocation attacks the C4 position of tryptophan. Though not expected to be the favored site of attack because it is meta to the indole nitrogen, that position is activated for nucleophilic attack by resonance involving the nitrogenous five-membered ring. Specificity for the C-4 carbon is likely due to positioning of the substrates in the active site of the enzyme.

Although several previous reports claimed that the enzyme had been purified, Gebler and Poulter (1992) appear to have been the first to fully characterize the activity of the purified DMAT synthase. The enzyme was purified from *Claviceps fusiformis* ATCC 26245 [erroneously annotated in type specimen collections as a *C. purpurea* strain (Pažoutová and Tudzynski, 1999)]. The monomeric size was estimated at 53 kDa, and by gel filtration analysis the native enzyme was determined (at 105 kDa) to be a homodimer. Unlike other prenyltransferases, no metal ion requirement has been noted. However, when assayed in a buffer with 4 mM Ca\(^{2+}\), the purified protein gave a specific activity of 500 nmol/min/mg, essentially the same as with 4 mM Mg\(^{2+}\), but approximately twice that of the measured \(V_{\text{max}}\) without added divalent cations and with the chelator EDTA included in the assay buffer. These divalent metal cations eliminated negative cooperativity of substrate binding observed both for dimethylallyl diphosphate and L-tryptophan, indicating that Ca\(^{2+}\) and Mg\(^{2+}\) probably had allosteric effects. In buffer with 4 mM MgCl\(_2\) the \(K_M\) for dimethylallyl diphosphate was 8 \(\mu\)M, and the \(K_M\) for L-tryptophan was 12 \(\mu\)M. The enzyme product was authenticated by mass spectrometry, UV spectrometry, and \(^1\)H-NMR.

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**Figure 4** Electrophilic aromatic substitution, constituting the most likely mechanism for DMAT synthase. The diphosphate moiety dissociates to generate the allylic carbocation, which then attacks the activated carbon-4 of the indole ring system. Approach of the aromatic C-4 is opposite the diphosphate leaving group, as indicated by experiments with specifically mono-tritiated (T) DMAPP (Shibuya et al., 1990).
5.1.2. DMAT Synthase Genes

The identity of DMAT synthase as a 53-kDa polypeptide with no primary sequence similarity to other prenyltransferases was substantiated by cloning of the gene, \textit{dmaW}. The same \textit{C. fusiformis} protein that had been purified by Gebler and Poulter (1992) was fragmented with cyanogen bromide, and N-terminal sequences of three released peptides were determined by Edman degradation. Based on these sequences, oligonucleotide primers were used to clone, by polymerase chain reaction (PCR)-based procedures, the cDNA and genomic DNA encoding this protein in \textit{C. fusiformis} (Tsai et al., 1995). The \textit{dmaW} cDNA was then expressed in yeast in both sense and, as control, antisense configurations relative to the yeast \textit{GAL4} promoter. Only the sense constructs resulted in activity expressed from galactose-induced yeast cells. Again, the authentic enzyme product was verified as 4-(\gamma,\gamma\text{-dimethylallyl})-tryptophan by MS and UV spectrometry, as well as by co-migration in HPLC with the previously verified standard.

Additional \textit{dmaW} genes have been cloned from \textit{C. purpurea} (Tudzynski et al., 1999; Wang, 2000), \textit{Neotyphodium} spp. (Wang, 2000), and \textit{Balansia obtecta} (Wang, 2000). Although there are disparate reports regarding the size of DMAT synthase from various \textit{Claviceps} species and isolates, the genes predict similar sizes for those of \textit{C. purpurea} and \textit{C. fusiformis}, as well as those from \textit{Neotyphodium} spp. and \textit{B. obtecta}. It is possible, of course, that the proteins are processed posttranslationally and thereby substantially reduced in size in some strains. The distributions of conserved regions over nearly the entire length of these genes argues against this possibility. The size estimated for the protein isolated from \textit{C. fusiformis} is in excellent accord with the \textit{dmaW} gene from the same isolate (Tsai et al., 1995).

Interestingly, although an aspartate-rich region is evident in the primary sequences of farnesyl diphosphate and geranylgeranyl diphosphate synthases (Song and Poulter, 1994), and such a region is proposed to be responsible for separation of the allyl and diphosphate moieties (Gebler and Poulter, 1992), no such region is conserved among DMAT synthases in \textit{C. fusiformis} (Tsai et al., 1995) and \textit{C. purpurea} (Tudzynski et al., 1999), nor among the DMAT synthase genes of endophytes (Wang, 2000).

The \textit{dmaW} gene of \textit{Neotyphodium} sp. Lp1, the hybrid endophyte of perennial ryegrass, has been disrupted by insertion of a modified hygromycin phosphotransferase gene (C. Machado, J. Wang, C. L. Schardl, unpublished data). To test whether the disruption eliminated ergot alkaloid biosynthesis, it was necessary to introduce the disruptant into its host plant and analyze the resulting symbiota. This lengthy procedure is necessary because production of ergot alkaloids in endophyte cultures is highly unreliable. The disruption of \textit{dmaW} caused no discernible reduction in compatibility of the endophyte with its
host plant. Symbiota containing the \textit{dmaW} disruptant or the disruptant complemented with the \textit{C. fusiformis dmaW} under control of a constitutive promoter from the \textit{Epichloë typhina tub2} gene were analyzed, confirming the role of \textit{dmaW} in ergot alkaloid biosynthesis (DG Panaccione and C Machado, unpublished data).

5.2. Peptide Synthetases

5.2.1. Biochemistry

Peptide synthetases, in general, are large, multifunctional enzymes containing a series of semiautonomous catalytic modules (reviewed by Marahiel et al., 1997; von Döhrren et al., 1997). Separate modules for each amino acid (or other carboxy acid) component of a peptide are arranged in series at regular intervals over the length of the polypeptide (Fig. 5). In most eukaryotic peptide synthetases, one large polypeptide contains all the modules and associated activities required to assemble the peptide. However, in several, typically prokaryotic, peptide synthetases, activities are divided between two polypeptides. The substrate specificity of each module from amino to carboxy terminus of the enzyme is co-linear with the structure of the final peptide product.

A typical peptide synthetase module is approximately 650 amino acids long and contains functional domains for recognizing and activating the substrate amino acid (or other carboxy acid) via adenylation (the adenylation domain), and for attaching the activated substrate as a thioester to a covalently bound thioester.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Representation of a general, four-module peptide synthetase. Each basic module consists of an adenylation domain (A) and a thiolation domain (T). Condensation domains (C) between modules form peptide bonds such that the peptide grows from amino to carboxy, with growing chain transferred to the amino acid bound to the next module. The substrate specificity of the individual modules is manifested in the amino acid (aa) sequence of the final peptide product.}
\end{figure}
pantethenate carrier (thiolation domain) (Fig. 5). Between modules is a region of approximately 450 amino acids that generally shares less sequence identity among peptide synthetases. This intermodule region was formerly referred to as a “spacer” but more recently has been demonstrated to function as a condensation domain that catalyzes peptide bond formation between amino acid substrates attached to adjacent thiolation domains of their respective modules (Gehring et al., 1998; Stachelhaus et al., 1998).

Some nonribosomally synthesized peptides recognize and incorporate carboxy acids other than amino acids into nonribosomally synthesized peptides (Marahiel et al., 1997; von Döhren et al., 1997). Examples of non-amino acid substrates include 2-hydroxyvaleric acid in AM-toxin and enniatin, 2,3-dihydroxybenzoic acid in enterobactin, and lysergic acid in the ergopeptines. The incorporation of non-amino acids into peptides appears to be catalyzed by typical peptide synthetase modules (Haese et al., 1993; Gehring et al., 1998; Johnson et al., 2000).

A C. purpurea peptide synthetase complex (lysergyl peptide synthetase, or LPS) that assembles ergopeptines from D-lysergic acid and three L-amino acids has been studied biochemically (Reiderer et al., 1996; Walzel et al., 1997). LPS of C. purpurea has activities and structural properties typical of other fungal peptide synthetases. However, it differs from all previously characterized eukaryotic peptide synthetases in having its activities divided between two distinct polypeptides: LPS1, which catalyzes the activation and thioesterification of the three L-amino acids, and LPS2, which catalyzes the activation and thioesterification of D-lysergic acid (Riederer et al., 1996). The activation of D-lysergic acid must occur before the other amino (or imino) acid constituents can be added to the growing peptide (Walzel et al., 1997). Only intermediates containing D-lysergic acid (e.g., D-lysergyl-L-alanine, D-lysergyl-L-alanyl-L-phenylalanine) have been released by chemically manipulating in-vitro LPS reactions, demonstrating that the synthesis of ergotamine starts with lysergic acid and the chain grows from amino to carboxy terminus (Walzel et al., 1997). In vitro, the final product is D-lysergyl-L-alanyl-L-phenylalanyl-L-proline lactam (Riederer et al., 1996). This intermediate has never been detected in vivo, presumably because it is immediately hydroxylated (at the α-carbon of L-alanyl residue) and cyclized to form the final cyclol ring of ergopeptines (Fig. 3). An isomer of this lactam containing D-proline (i.e., D-lysergyl-L-alanyl-L-phenylalanyl-D-proline lactam) has been detected in cultures of C. purpurea (Stütz et al., 1973; Floss, 1976) and also among the reaction products of LPS (Riederer et al., 1996). Its accumulation in cultures indicates that D-proline-containing lactam cannot be cyclized via the hydroxylated intermediate (Riederer et al., 1996).

Some of the modules in LPS1 recognize alternative substrates supplied in vitro (Riederer et al., 1996). For example, L-2-aminobutyric acid or L-valine
could be substituted for L-alanine at amino acid position I, and L-leucine could be substituted for L-phenylalanine at amino acid position II. Each of these substitutions occurs in natural ergopeptines produced by *C. purpurea* (Fig. 3). These data indicate that the various ergopeptines isolated from clavicipitaceous fungi may be the products of a single enzyme complex, and that cellular pools of L-amino acids may dictate, in part, the abundance of specific ergopeptines produced.

### 5.2.2. Lysergyl Peptide Synthetase Genes

A gene designated *cpps1* (for *C. purpurea* peptide synthetase 1) and capable of encoding a three-module peptide synthetase, has been found tightly linked to a gene encoding DMAT synthase in *C. purpurea* isolate P1 (Tudzynski et al., 1999). The tri-modular nature of the deduced product of this gene, as well as its clustering with a DMAT synthase gene, indicate that *cpps1* encodes LPS1. Moreover, in the deduced amino acid sequence of *cpps1* there is a series of 17 amino acids that is a near match to a tryptic fragment obtained from purified LPS1 (Tudzynski et al., 1999). *Claviceps purpurea* *cpps1* contains at least one intron, which is unusual among peptide synthetase genes, even those from eukaryotes. Among the published peptide synthetase gene sequences, only one other peptide synthetase gene (interestingly, from the clavicipitaceous entomopathogen *Metarhizium anisopliae*) is known to contain an intron (Bailey et al., 1996).

As described above, the *C. purpurea* LPS complex is unique among eukaryotes in having its activities divided between two different polypeptides. In its two-polypeptide nature, LPS resembles some prokaryotic peptide synthetases such as tyrocidine synthetase and gramicidin synthetase. However, unlike the prokaryotic peptide synthetases consisting of two polypeptides, the available DNA sequence of *cpp1* (Tudzynski et al., 1999) indicates that its product does not begin with a recognizable condensation domain, as would be typical of the “receiving” synthetase of a dual-polypeptide system (Marahiel et al., 1997; von Döhren et al., 1997).

Independent of the work of Tudzynski and colleagues, sequences encoding LPS have been identified in other isolates of *C. purpurea* and in the perennial ryegrass endophyte *N. lolii* (Panaccione et al., 2001). A peptide synthetase gene fragment (*Cp605*), previously amplified from *C. purpurea* isolate ATCC 34501 by a PCR approach (Panaccione, 1996), was found to be clustered with the DMAT synthase gene *dnaW* in *Claviceps purpurea* isolate ATCC 20102 (Panaccione et al., 2001). The cluster isolated from *C. purpurea* ATCC 20102 had tandem copies of the peptide synthetase gene linked to the DMAT synthase gene. The peptide synthetase genes from the different *C. purpurea* isolates appear highly similar based on hybridization analyses and partial DNA sequence analyses, and include a region highly similar to
the previously cloned peptide synthetase gene fragment Cp605 (Panaccione, 1996) in their final module.

To assess the function of the ergopeptine-associated peptide synthetase gene in a grass–Neotyphodium endophyte association, a gene (designated lpsA for lysergyl peptide synthetase) containing sequences similar to Cp605 was isolated from an available library of N. lolii. The N. lolii lpsA gene has a primary structure similar to that published for C. purpurea P1 cpps1 over most of its length, but appears to diverge near the amino terminus to contain additional functional domains not encoded by the C. purpurea gene (P. Damrongkool, R. D. Johnson, and D. G. Panaccione, unpublished data). The N. lolii gene was used to direct a gene knockout in the closely related fungus Neotyphodium sp. Lp1, instead of N. lolii, because of Lp1’s faster growth and reliable production of ergovaline. Strains containing the lpsA knockout were able to colonize host plants as well as wild-type Lp1 or strains containing an ectopically integrated gene knockout construct, as assessed by immunoblotting and quantitative PCR. However, the perennial ryegrass associations containing the lpsA knockout strain did not produce ergovaline or other ergopeptines (Panaccione et al., 2001).

Blocking ergopeptine production by knockout of the peptide synthetase step provides a means to eliminate ergovaline from the grass–endophyte symbiosis for basic studies and, potentially, to ameliorate the livestock toxicoses associated with ergovaline. The knockout at this particular pathway step also presents an interesting opportunity to study regulation of the ergot alkaloid biosynthetic pathway. HPLC analyses of the symbiota containing the lpsA knockout fungus did not reveal any new peaks of an appreciable size. Interestingly, blocking this relatively late step in the pathway does not result in excessive accumulation of more polar simple amides of lysergic acid in analyses conducted to date (Panaccione et al., 2001; R. D. Johnson and D. G. Panaccione, unpublished data). This observation is consistent with early biochemical data indicating feedback inhibition of chanoclavine I cyclase by lysergic acid and elymoclavine (Erge et al., 1973, as cited in Floss, 1976).

All ergopeptine-producing fungi examined thus far contain DNA that hybridizes with lpsA of N. lolii, whereas those that are reported to not produce peptide ergot alkaloids do not yield detectable signals upon hybridization with this gene (Panaccione et al., 2001). Among the producers found to contain hybridizing sequences are C. purpurea, Epichloë festucae, N. coenophialum, and, of course, N. sp. Lp1 and N. lolii. Ergopeptine nonproducers without hybridizing sequences include C. fusiformis, E. typhina, and the entomopathogen Cordyceps militaris. A similar absence of hybridizing sequences in ergopeptine nonproducers has also been found with probes prepared from the C. purpurea gene (Panaccione, unpublished data).
5.2.3. Other Peptide Synthetase Genes and Hypothetical Peptide Synthetases

Early feeding experiments with labeled alanine indicate that the nonlysergyl components of ergonovine and lysergic acid-2-hydroxyethylamide may be derived from alanine (Floss, 1976, and references cited therein). If so, the lysergyl-alanine precursor would likely be the product of a peptide synthetase. However, Floss (1976) argued against lysergyl-alanine as a precursor to ergonovine, based on the failure to isolate or trap this intermediate from ergot alkaloid-producing cells. In retrospect, the lack of free lysergyl-alanine may be explicable if such a precursor were reduced prior to or during release from its covalent attachment to the hypothetical peptide synthetase. Many peptide synthetases carry “tailoring” domains to N-methylate or epimerize bound amino acid substrates (Marahiel et al., 1997; von Döhren et al., 1997). Although reductase activity has not yet been identified as a tailoring domain in a peptide synthetase, modular polyketide synthases from fungi certainly carry such semiautonomous reductase domains (e.g., Yang et al., 1996; Brown et al., 1999; Tkacz, 2000). A lysergyl-alanine precursor to the simple amides of lysergic acid is attractive because it could be formed through an association of the lysergic acid-activating LPS2 with a single-module, alanine-activating peptide synthetase. The ability for LPS2 to donate lysergic acid to two different “receiving” peptide synthetases would provide a compelling explanation for the separation of LPS2 from the remaining modules in LPS1, which is otherwise unprecedented for a eukaryotic peptide synthetase. *Claviceps purpurea* is known to contain several additional peptide synthetase genes whose activities have not been established (Panaccione, 1996; Annis and Panaccione, 1998). An involvement for these, or any other, peptide synthetase gene in ergonovine biosynthesis could be addressed by gene knockout analyses.

5.3. Other Genes Hypothesized from *C. purpurea* Ergot Alkaloid Gene Cluster

The typical clustering of genes encoding secondary metabolite pathway genes in fungi (reviewed in Keller and Hohn, 1997) may provide a mechanism for the identification of additional genes involved in ergot alkaloid biosynthesis. At least some of the pathway genes appear to be clustered in *C. purpurea*. In addition to the peptide synthetase and DMAT synthase genes discussed in detail above, Tudzynski et al. (1999) also described two open reading frames with the ability to encode oxidoreductases. One of the oxidoreductases had a primary structure typical of FAD-containing oxidoreductases, leading Tudzynski et al. (1999) to hypothesize that this gene may encode chanoclavine I cyclase, which has been characterized as a FAD-containing oxidoreductase (Floss, 1976). An additional oxidoreductase in the *C. purpurea* P1 cluster is consistent with additional...
oxidation and reduction steps required in the conversion of the various clavines, and for additional steps in the pathway. Further chromosome walking and sequence comparisons are expected to reveal additional ergot alkaloid biosynthesis genes. The annotation of genes in the cluster (by comparison to sequence databases) may provide hypotheses for the catalysis of reactions in the ergot alkaloid pathway that have not been biochemically characterized.

Interestingly, in limited analyses to date, there is no evidence for clustering of genes encoding DMAT synthase and LPS in Neotyphodium spp. If the Neotyphodium spp. genes are clustered, then their spatial relationships would have to be different from those observed for the genes in the C. purpurea cluster (J. Wang, D. G. Panaccione, C. L. Schardl, unpublished data).

6. CONCLUSIONS

Research on the molecular genetics of ergot alkaloid biosynthesis holds promise to address significant problems in agriculture. The lpsA knockout strain of N. sp. Lp1 appears to produce simpler ergot alkaloids but not ergovaline. Successful knockout of dmaW should result in the loss of all ergot alkaloids. The symbiota containing these knockout strains, when compared with endophyte-free perennial ryegrass and that infected with wild-type Lp1, give us the tools to investigate the role of ergovaline and simpler ergot alkaloids in the various costs (toxicosis) and benefits (biotic and abiotic stress resistance) generally associated with endophyte infection of grasses. With these cloned genes, it should also be possible to identify and knock out homologs in other endophytes that have different alkaloid profiles, for a more complete understanding of ergot alkaloid roles relative to other endophyte alkaloids. In particular, N. coenophialum and N. sp. Lp1 differ in that the former produces the insecticidal loline alkaloids, whereas the latter does not. Therefore, eventual tests of fitness enhancement—particularly anti-insect and antinematode effects—in knockout mutants of both these endophytes will allow us to assess whether ergovaline is a significant contributor in the absence or perhaps even in the presence of lolines. Such information will be useful to forage and turf breeders because of the wide variation in alkaloid profiles among endophytes in the various economic grass species.

A central aim of manipulating the endophyte alkaloid profiles, particularly eliminating ergopeptines, is to reduce the potential for toxicosis to livestock. The strategy would involve replacing wild-type endophyte with the modified (dmaW or lpsA knockout) endophyte in breeding lines for cultivars of tall fescue and perennial ryegrass. Because all indications are that N. coenophialum and N. lolii transmit only vertically via seeds, and never spread contagiously, the breeding lines should maintain only the modified endophytes. An open question is whether such a modification will significantly affect longevity of the forage grass stands. It has been well established that N. coenophialum and N. lolii provide enhanced

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stand longevity in pastures, but the underlying reasons are unclear. Any or all of
the various benefits conferred by these endophytes may be responsible, but the
relative importance of grazing resistance is unknown. If animals are capable of
detecting and selectively grazing plants that are low in ergot alkaloids, they may
cull both endophyte-free plants and those with endophytes that fail to synthesize
these alkaloids.

Whereas the recent molecular genetic studies described herein have been
facilitated by previous biochemical investigations, further molecular genetic
analyses may help elucidate the nature and regulation of the biochemical
pathway. Sequence analysis of genes linked to known ergot alkaloid biosynthesis
genes and annotation of their likely function may provide novel information
about pathway enzymes and intermediates. The ability to block the pathway by
gene knockout may facilitate the characterization of difficult intermediates or
studies of pathway regulation.

Manipulation of ergot alkaloid biosynthesis genes may also provide
opportunities to engineer novel metabolites. Ergot alkaloids are used clinically
for treatment of migraines, Parkinson’s disease, hypertension, and depression
(e.g., Brown et al., 1991; Markstein et al., 1992; Muck-Seler and Pericic, 1993;
Ohno et al., 1994). Moreover, they are useful tools for basic research on serotonin
receptors in the central nervous system (e.g., Markstein et al., 1992; Sundaram
et al., 1993). In theory, ergopeptines with novel amino acid sequences in the
tripeptide moiety could be generated by substitution of modules with specificities
for different amino acids for one or more of the modules in LPS. Such an
approach has been successful in generating novel nonribosomally synthesized
peptides in bacterial systems (Stachelhaus et al., 1995; Schneider et al., 1998).

Finally, comparison of the sequences of ergot alkaloid biosynthesis genes
from different clavicipitaceous fungi and the arrangement of these genes in their
respective genomes may provide information about the evolution of the
Clavicipitaceae and toxicogenic ability within the family. The research being
conducted currently indicates that there will be enough similarity between the
pathway genes in \textit{Claviceps} and \textit{Neotyphodium/Epichloë} species so that progress
in one fungus will facilitate progress in others, but enough differences to make
the comparisons interesting.

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Genetic Manipulation of Clavicipitalean Endophytes

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Massey University, Palmerston North, New Zealand

1. INTRODUCTION

*Epichloë* endophytes are an important group of clavicipitaceous fungi (Clavicipitaceae, Ascomycota) that form symbiotic associations with temperate grasses of the subfamily Pooideae. They include the sexual *Epichloë* species and their asexual anamorphic derivatives, the *Neotyphodium* species (Schardl, 1996a). These biotrophic fungi colonize the intercellular spaces of leaf primordia, leaf sheaths, and culms of vegetative tissues (Hinton and Bacon, 1985; Philipson and Christey, 1986). The asexual species form asymptomatic mutualistic associations with their host and are transmitted vertically through the seed following colonization of the developing ovule (Philipson and Christey, 1986). The sexual species behave as mutualists during the vegetative phase of the plant growth cycle, but development of the floral meristem can trigger epiphytic growth of the fungus and formation of a stroma around the flag leaf, which “choke” emergence of the floral meristem. Species that form highly antagonistic associations, in which most of the tillers are sterilized, are only transmitted horizontally (Schardl and Leuchtmann, 1999). Other species form pleiotropic associations, in which some tillers show choke and others remain asymptomatic (Leuchtmann and...
Schardl, 1998; Leuchtmann et al., 1994). In nature, the mating system is heterothallic (outcrossing) and is mediated by a third symbiotic partner, an anthomyiid fly (*Botanophila* sp.) (Bultman et al., 1995; White and Bultman, 1987). The major benefits for grass endophytes are access to nutrients from the host apoplast and, in the case of the mutualists, a means of dissemination through the seed. Biological benefits to the host include protection from insect (Siegel et al., 1990) and mammalian herbivory (Bacon et al., 1977), resistance to nematodes (Kimmons et al., 1990) and some fungal pathogens (Gwinn and Gavin, 1992), drought tolerance (Arachevaleta et al., 1989), and greater field persistence (Hill et al., 1990; West et al., 1988).

2. GENETIC STRUCTURE OF *EPICHLOË* ENDOPHYTES

The genus *Epichloë* comprises 10 biological species of endophytic fungi (Schardl and Wilkinson, 2000). These correspond to nine distinct mating populations, five of Eurasian origin—*E. typhina*, *E. festucae*, *E. baconii*, *E. bromicola*, and *E. sylvatica*—and four of North American origin—*E. elymi*, *E. amarillans*, *E. glyceriae*, and *E. brachyelytri* (Schardl and Wilkinson, 2000). The tenth species, *E. clarkii*, is a distinct morphospecies that falls within the same mating population, MPI, as *E. typhina*. The biological species groupings identified by mating tests are strongly supported by both biochemical and molecular data. Members of the same species form clusters of genetically related individuals based on their isozyme genotypes and DNA sequences of the noncoding regions of the β-tubulin (*tub2*) and the internal transcribed spacers (ITS) of the nuclear ribosomal RNA (*rrn*) genes (Fig. 1). Using these methods it has been established that *Neotyphodium* species evolved from the *Epichloë* species, either directly from a single species, as

![Image](https://example.com/image.png)

**Figure 1** Structure of *Epichloë* endophyte ribosomal RNA (rDNA) and β-tubulin (*tub2*) genes.
appears to be the case for *N. lolii*, or more commonly by interspecific hybridization (Scharldl et al., 1994; Tsai et al., 1994). The presence of multiple copies of *tub2*, *pyr4* (orotidine-5'-monophosphate decarboxylase) and several other genes in the asexual hybrids suggests that these species frequently retain the genome complements of their parents (Scharldl et al., 1994; Collett et al., 1995). The one exception is the rDNA locus—all hybrids examined to date contain a unique sequence (Scharldl et al., 1994; Tsai et al., 1994; Moon et al., 2000). It has been proposed that the presence of just one parental rDNA sequence is the result of homogenization processes associated with the concerted evolution of this multigene family (Ganley and Scott, 1998, 2001). Because of this limitation, additional intron sequences, such as those from the highly conserved actin (*act1*) and translation elongation factor 1-α (*tef1*) genes, have been used to analyze phylogenetic relationships of clavicipitalean endophytes (Craven et al., 2001).

### 3. GENOMES OF *EPICHLÖE* ENDOPHYTES

Estimates of the genome size of grass endophytes range from around 28 Mb, for the haploid sexual species, and up to 58 Mb for some of the interspecific hybrids (Kuldau et al., 1999). Electrophoretic karyotypes are consistent with these estimates, with between 5 and 7 chromosomes in the haploid species and between 10 and 14 chromosomes in the hybrids (Fig. 2). The sizes of these chromosomes range from 0.9 to 10.5 Mb (Kuldau et al., 1999; Murray et al., 1992). The ability

![Figure 2](image-url)

**Figure 2** Electrophoretic karyotypes of *Epichloë* endophytes. Electrophoresis conditions are as described by Murray et al. (1992). Lane 1, *Saccharomyces cerevisiae*; lane 2, *Schizosaccharomyces pombe*; lane 3, *Epichloë typhina* × *Neotyphodium lolii* strain Lp1; lane 4, *E. typhina* × *N. lolii* strain Lp1-1; lane 5, *E. typhina* × *N. lolii* strain Lp1-2; lane 6, *N. lolii* strain Lp5; lane 7, *N. lolii* strain Lp5-1; lane 8, *N. lolii* strain Lp19; lane 9, *N. lolii* strain Lp20; lane 10, *N. lolii* strain Lp21.
to resolve these large chromosomes will allow the construction of chromosome-specific bacterial artificial chromosome (BAC) and cosmid libraries, as has been carried out for *Aspergillus nidulans* and *Magnaporthe grisea* (Zhu et al., 1997; Kupfer et al., 1997; Brody et al., 1991). BAC and cosmid libraries will be useful genetic resources for constructing physical maps to endophyte genomes and in providing templates for whole-genome sequence analysis. An additional component of the endophyte genome is the mitochondrion chromosome. Inheritance of the mitochondrial genome is predominantly maternal, but nonparental genomes are sometimes found, suggesting that heteroplasmy occurs during sexual reproduction, thereby allowing recombination between parental genomes (Chung et al., 1996).

Two types of parasitic elements are found in clavicipitalean endophytes—linear DNA plasmids and double-stranded RNA viruses (Mogen et al., 1991; Zabalgozcoa et al., 1998). Linear plasmids are resolved from linear chromosomal DNA when undigested genomic DNA is separated by agarose gel electrophoresis (Fig. 3). *E. typhina* has been shown to contain three plasmids, designated Callan-a (7.5 kb), Aubonne-a (2.1 kb), and Bergell (2.0 kb) (Chung et al., 1996; Mogen et al., 1991). These plasmids share little homology with one another and, like the mitochondrial genome, show predominantly maternal inheritance (Chung et al., 1996). Two dsRNA elements have been identified in *E. festucae* (Zabalgozcoa et al., 1998). Both RNA species were associated with isometric virus-like particles of 50 nm in diameter, strongly suggesting that they are encapsulated. The presence of these Totiviridae viruses had no effect

![Figure 3](image.png)

**Figure 3**  Linear plasmids in *Epichloë typhina*. Lane 1, *λ-HindIII* size markers; lanes 2–4, undigested *E. typhina* strain E8 DNA.
on the growth rates or colony morphology of the *E. festucae* in culture when compared with strains without the viruses.

While very little is known about the composition of endophyte genomes, repetitive elements, including retroelements (Young and Scott, unpublished results) and microsatellites (Moon et al., 1999; Groppe et al., 1995), are abundant components of these genomes. Southern hybridization analysis demonstrates that retroelement-like sequences are present in high copy number in the genomes of

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**FIGURE 4** Retroelement sequences in *Epichloë* endophyte genomes. Southern hybridization of *Epichloë* genomic DNA (2 μg) digested with *Eco*RI (lanes 2–4) and *Hind*III (lanes 5–7) and probed with a retroelement sequence, lol-4/23. Lane 1, λ-*Hind*III size markers; lanes 2 and 5, *N. lolii* strain Lp19; lanes 3 and 6, *E. festucae* strain F11; Lanes 4 and 7, *E. festucae* strain E189.
E. festucae and N. lolii and are highly dispersed (Fig. 4). The abundance of these elements may provide “hot spots” for recombination, generating mutational events such as chromosome rearrangements and deletions. Genome changes of this type may be responsible for the colony morphology instability that is frequently observed when N. lolii is isolated from plant tissue (Christensen et al., 1991).

Microsatellites, also known as simple sequence repeats (SSR), are abundant components of eukaryotic genomes and are found in both coding and noncoding regions of fungal genomes (Field and Wills, 1998). They form tandem arrays of DNA motifs (of 1–10 nucleotides) that are hypervariable in length as a result of DNA replication slippage processes. Microsatellites are therefore very useful molecular markers for a range of applications. The utility of using microsatellite loci to detect Epichloë endophytes in grass tissues was first demonstrated by Groppe and colleagues, using polymerase chain reaction (PCR) assay based on an AAG-containing locus identified in E. bromicola (Groppe et al., 1995; Groppe and Boller, 1997). Subsequently, Moon et al. (1999) developed a microsatellite-based multiplex assay with automated analysis using fluorescently labeled primers for detection. This method of analysis provides a rapid and sensitive approach to identifying grass endophytes, using DNA template prepared from either mycelium or grass tissues. The specificity of this assay to detect endophyte sequences in a host background has made it possible to generate microsatellite profiles of unculturable endophytes, such as the recently described N. occultans, found in annual ryegrasses (Moon et al., 2000). Table 1 summarizes the microsatellite sequences identified to date in the genomes of Epichloë endophytes.

4. ENDOPHYTE METABOLITES AND HOST PROTECTION

The ability of Epichloë endophytes to synthesize a range of secondary metabolites in planta constitutes a major ecological benefit for the symbiosis (Schardl, 1996b). Peramine and N-formylloline are potent deterrents of insect feeding, whereas lolitrem B and ergovaline are antimammalian compounds of the indole-diterpene and ergot-alkaloid groups of mammalian toxins (Bush et al., 1997). While synthesis of mammalian toxins may be ecologically beneficial, from an agricultural perspective endophyte synthesis of these toxins is detrimental to grazing livestock. The lolitrems are potent tremorgenic neurotoxins implicated in “ryegrass staggers” in sheep (Fletcher and Harvey, 1981; Gallagher et al., 1981), and the ergot alkaloid toxins are implicated in “fescue toxicosis” in cattle (Bacon et al., 1977). Consequently, there is considerable interest in selecting for host–endophyte associations that minimize the detrimental effects of mammalian toxins to grazing animals yet retain the protective benefits, such as synthesis of anti-insect compounds, that inter alia,
<table>
<thead>
<tr>
<th>Locus</th>
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<th>Reference</th>
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<tr>
<td>B1</td>
<td>(AAG)$_{18}$</td>
<td><em>E. bromicola</em></td>
<td>NF 62A</td>
<td>Groppe et al., 1995</td>
</tr>
<tr>
<td>B2</td>
<td>(AC)$_{18}$</td>
<td><em>E. typhina</em></td>
<td>E8</td>
<td>Moon et al., 1999</td>
</tr>
<tr>
<td>B3</td>
<td>$C_{12}$&amp; (GA)$_7$</td>
<td><em>E. typhina</em></td>
<td>E8</td>
<td>Moon et al., 1999</td>
</tr>
<tr>
<td>B4</td>
<td>(CA)$_3$GCG(CA)$_3$ACG(CA)$_3$A(CA)$_4$</td>
<td><em>E. typhina</em></td>
<td>E8</td>
<td>Moon et al., 1999</td>
</tr>
<tr>
<td>B5</td>
<td>(CATCTCATCA)$_5$</td>
<td><em>E. typhina</em></td>
<td>E8</td>
<td>Moon et al., 1999</td>
</tr>
<tr>
<td>B6</td>
<td>(CAT)$_2$CAC(CAT)$_3$</td>
<td><em>E. typhina</em></td>
<td>E8</td>
<td>Moon et al., 1999</td>
</tr>
<tr>
<td>B7</td>
<td>(AG)$_5$A(AG)$_3$</td>
<td><em>E. typhina x N. lolii</em></td>
<td>Lp1</td>
<td>Moon et al., 1999</td>
</tr>
<tr>
<td>B8</td>
<td>(AC)$_8$T$_6$</td>
<td><em>E. typhina x N. lolii</em></td>
<td>Lp1</td>
<td>Moon et al., 1999</td>
</tr>
<tr>
<td>B9</td>
<td>G$_9$AG$_9$ &amp; (GAGAG)$_2$C(GAGGA)$_2$</td>
<td><em>E. typhina x N. lolii</em></td>
<td>Lp1</td>
<td>Moon et al., 1999</td>
</tr>
<tr>
<td>B10</td>
<td>(AGC)$_3$CG(CAT)$_3$C(AA)$_5$</td>
<td><em>E. typhina x N. lolii</em></td>
<td>Lp1</td>
<td>Moon et al., 1999</td>
</tr>
<tr>
<td>B11</td>
<td>(GACA)$_{18}$</td>
<td><em>N. lolii</em></td>
<td>Lp19</td>
<td>Moon et al., 1999</td>
</tr>
<tr>
<td>B12</td>
<td>(CAG)$_{12}$</td>
<td><em>N. lolii</em></td>
<td>Lp19</td>
<td>Young &amp; Scott, unpub</td>
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<tr>
<td>B13</td>
<td>(CTT)$_3$(TATA)$_2$(CATA)$_2$TATA</td>
<td><em>N. lolii</em></td>
<td>Lp19</td>
<td>McGill &amp; Scott, unpub</td>
</tr>
</tbody>
</table>
contribute to field persistence of the host grass (Fletcher and Easton, 2000; Bouton, 2000).

The molecular cloning of genes in these secondary metabolite pathways should therefore provide a set of molecular tools to identify naturally occurring endophyte strains that lack the ability to synthesize these toxins. Alternatively, it will be possible to genetically block these toxin pathways by deleting genes that are involved in the early steps using gene-replacement methodology (Rothstein, 1991).

5. MOLECULAR CLONING OF INDOLE-DITERPENE BIOSYNTHESIS GENES

Indole-diterpenes are a structurally diverse group of fungal secondary metabolites, many of which are potent tremorgenic mammalian toxins (Steyn and Vleggaar, 1985). While the chemical complexity of these compounds is well documented, very little is known about the nature of the biochemical intermediates or the enzymology of their biosynthesis. The biosynthetic schemes proposed for the synthesis of this class of compounds are based on radiolabeling studies and comparisons of the structures of various indole-diterpenes isolated from a range of fungi (Mantle and Weedon, 1994; Munday-Finch et al., 1996). These schemes propose that geranylgeranyldiphosphate (GGPP), derived from mevalonic acid, and indole, derived from tryptophan, are the primary precursors for indole-diterpene synthesis. However, recent work on the biosynthesis of paxilline in Penicillium paxilli (Young et al., 2001) and nodulisporic acid in Nodulisporium sp. (Byrne et al., 2002), has shown that the synthesis of GGPP occurs by a pathway specific GGPP synthase, and that indole is derived from anthranilic acid rather than tryptophan. The discovery of a paxilline-specific GGPP synthase in P. paxilli suggests that IPP (C5), rather than GGPP (C20), is the primary carbon precursor for indole-diterpene biosynthesis (Young et al., 2001). The high rates of incorporation of radiolabelled anthranilic acid, compared to tryptophan, into nodulisporic acid (Byrne et al., 2002), suggest that indole-3-glycerolphosphate is the primary source of the indole group for this class of compounds (Fig. 5).

The recent cloning of a cluster of genes from P. paxilli for the biosynthesis of paxilline provides for the first time an insight into the number of genes required for the synthesis of an indole-diterpene, and the likely biochemical function of the enzymes involved. Sequence analysis identifies genes with similarities to a GGPP synthase (paxG), a FAD-dependent monooxygenase (paxM), a prenyltransferase (paxC), and two cytochrome P450 monooxygenases (paxP, and paxQ). Deletions of paxG (47) and paxM (McMillan, Young, and Scott, unpublished results) are unable to synthesize paxilline and any other known indole-diterpene, suggesting that the enzymes encoded by these genes are
required for early steps in the pathway (Fig. 5). Deletion derivatives of *paxP* and *paxQ* accumulate paspaline and 13-desoxypaxilline (McMillan, Carr, Young, and Scott, unpublished results), compounds previously identified in *P. paxilli* and proposed intermediates in a metabolic grid for the biosynthesis of paxilline (Munday-Finch et al., 1996).

Because GGPP synthase is a relatively well conserved gene, it has been possible to design degenerate primers to conserved regions of *P. paxilli* PaxG and other fungal GGPP synthases, that amplify by PCR the *N. lolii* ortholog, *ltmG*,

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**ERGOT ALKALOID BIOSYNTHESIS**

DMAPP → DMAT → CLAVINES → ERGOVALINE

**INDOLE DITERPENE BIOSYNTHESIS**

DMAPP → IPP → PAXILLINE

DMAPP → GGPP → PASPALINE → 13-DESOXYPAXILLINE → LOLITREM B

**FIGURE 5** Proposed pathways for ergot alkaloid and indole-diterpene biosynthesis in clavicipitalean endophytes. Ergovaline is synthesized from DMAP and tryptophan via a DMAT synthase (*dmaW* in *N. lolii/cpd1* in *Claviceps purpurea*) and a nonribosomal peptide synthase (*ipsA/cpps1*). Paxilline and lolitrem B are synthesized from DMAPP, IPP, and indole-3-glycerol phosphate via a GGPP synthase (*paxG* in *P. paxilli/ltmG* in *N. lolii*), a FAD-dependent monooxygenase (*paxM/ltmM*), and a cytochrome P450 monooxygenase (*paxP/ltmP*).
of this gene (Young and Scott, unpublished results). Adjacent to \(ltmG\) are orthologs of \(paxM\) (\(ltmM\)) and \(paxP\) (\(ltmP\)). Confirmation that these three genes are necessary for lolitrem biosynthesis awaits targeted deletion of the genes and analysis of the ability of the corresponding transformants to synthesize lolitrems \textit{in planta}.

6. MOLECULAR CLONING OF ERGOT ALKALOIDS

The biosynthesis of the ergot alkaloids is the best understood of the endophyte alkaloid biosynthetic pathways, principally because of the research carried out on \textit{Claviceps purpurea}, the fungus responsible for St. Antony’s fire (ergotism) from contaminated rye (Socic and Gaberc-Porekar, 1992). The first step in this pathway is the synthesis of dimethylallyltrypotphan (DMAT), a reaction catalyzed by DMAT synthase (Fig. 5). The substrates for this reaction are dimethylallyldiphosphate (DMAPP), a derivative of mevalonic acid, and tryptophan (Gebler and Poulter, 1992). DMAT is converted via several intermediates to clavine alkaloids, such as lysergic acid, that are characterized by the presence of an ergolene ring system. The ergolene acids (e.g., lysergic acid) and ergolene alcohols undergo further transformations to form more complex ergopeptide derivatives such as ergotamine. The synthesis of ergotamine is catalyzed by two nonribosomal peptide synthases, LPS1 (lysergyl peptide synthase 1) and LPS2, which sequentially add the amino acids alanine, phenylalanine, and proline to the activated lysergic acid (Fig. 5) (Riederer et al., 1996; Walzel et al., 1997). The structure of ergovaline predicts that the endophyte peptide synthetase (PS) responsible for the synthesis of this metabolite will be very similar to LPS1 but with a second module that recognizes valine rather than phenylalanine.

The first ergot alkaloid biosynthetic gene to be cloned was the \textit{Claviceps fusiformis} DMAT synthase (\textit{dmaW}) (Tsai et al., 1995). Subsequently a cluster of genes, including a DMAT synthase (\textit{cpd1}) and nonribosomal peptide synthase (\textit{cpps1}), were isolated from \textit{Claviceps purpurea} (Tudzynski et al., 1999). The 100\% sequence identity observed between a 17-amino acid tryptic peptide of module 2 of LPS1 and the corresponding deduced polypeptide sequence of CPPS1 is good evidence that \textit{cpps1} encodes a peptide synthetase for the synthesis of ergotamine (Tudzynski et al., 1999). Using a PCR approach, Panaccione (1996) succeeded in amplifying three unique PS sequences from \textit{C. purpurea} and \textit{Neotyphodium coenophialum}. Each of the \textit{N. coenophialum} genes was present in 2–3 copies, a result consistent with the tri-parental hybrid origin of this particular taxonomic group (Tsai et al., 1994). One of the \textit{C. purpurea} sequences, Cp605, was shown to cross-hybridize to \textit{N. coenophialum} and to be on the same cosmid that contains the DMAT synthase. These results indicated that the endophyte ortholog of Cp605 was a strong candidate for an ergovaline peptide synthase.
This was recently demonstrated by using Cp605 as a heterologous probe, to isolate a *N. lolii* ortholog lpsA, which, when disrupted in strain Lp1 (*E. typhina × N. lolii*), fails to synthesize ergovaline *in planta* (Panaccione et al., 2001). This result provides genetic proof that the *N. lolii* peptide synthase is necessary for ergovaline biosynthesis and demonstrates for the first time that endophyte toxin biosynthesis can be specifically blocked by targeted disruption of a gene in the pathway.

7. **MOLECULAR BREEDING OF ENDOPHYTES**

The cloning of genes for indole-diterpene (Young et al., 2001) and ergot alkaloid biosynthesis (Tudzynski et al., 1999; Panaccione et al., 2001) reveals, for the first time, the identity of the genes involved, and the likely biochemical function of the gene products. Targeted disruption of individual genes and chemical analysis of the product(s) that accumulates establishes a role for the enzyme in the pathway and identifies the likely substrate for that enzyme (Young et al., 2001; Panaccione et al., 2001). Purification of the intermediate that accumulates in a mutant provides a source of the substrate to identify in vitro, using purified enzyme, the product of the enzyme reaction. Alternatively, individual genes in the pathway could be introduced into deletion derivatives lacking the entire pathway cluster, or into *Saccharomyces cerevisiae* (Tsai et al., 1995), and products identified by feeding radiolabelled intermediates to the transformants. This approach has recently been used successfully to dissect the steps involved in the biosynthesis of gibberellins in *Gibberella fujikuroi* (Rojas et al., 2001). Similarly, feeding purified intermediates to single-gene-deletion mutants establishes whether an intermediate is before or after a particular enzymatic step. These combined approaches will define the pathways for the synthesis of these secondary metabolites.

The availability of gene probes for toxin biosynthesis pathways will allow the screening of *Epichloë* endophytes for the presence and distribution of these genes. This “molecular selection” approach to identify strains that lack the genetic capacity to synthesize toxins will eliminate the uncertainty of current selection processes, which rely on detection of the toxin in the plant—a result that is dependent on host–genotype interactions and physiological effects. It is possible, however, that natural isolates will have the genes but still lack the ability to produce the toxins, regardless of the host genotype background. A precedent for this is the presence of genes for aflatoxin biosynthesis in *Aspergillus sojae* and *A. oryzae*, species which are used in Asian food fermentations (e.g., koji preparation) (Klich et al., 1995, 1997). These species are closely related to the aflatoxin producing *A. parasiticus* and *A. flavus*, respectively. The inability of *A. oryzae* isolates to synthesize aflatoxins is due to the absence of one or more of the genes in the pathway, but the molecular basis for the lack of expression in *A. sojae* is still not known. Using molecular
approaches it will be possible to verify that endophyte strains classified as toxin-negative, based on their chemical phenotype in the host, indeed lack a key gene in the pathway, or alternatively, are blocked in their ability to express those genes. These selection strategies will enhance the robustness of the tests for deciding whether an endophyte is toxin-negative or -positive and overcome the commercial risk of unexpected expression of a particular toxin when a strain, for example, is moved from one host background to another.

The cloning of genes for toxin biosynthesis also opens up the possibility of using recombinant DNA (genetic engineering) methods to disrupt an early step in the toxin biosynthetic pathway. This approach has already been used to totally block the synthesis of paxilline, and all known indole-diterpene intermediates, in *P. paxilli* by targeted deletion of the GGPP synthase, paxG, gene (Young et al., 2001). As described above, a similar approach has recently been used to disrupt the peptide synthase required for ergovaline biosynthesis in *Neotyphodium* sp. strain Lp1 (Panaccione et al., 2001). For endophytes that have the capacity to synthesize both ergot alkaloids and indole-diterpenes, it may be necessary to disrupt both pathways, as disruption of one pathway may lead to an increased flux of the common isoprenoid intermediates into the other pathway (see Fig. 5). This could result in an even higher level of one of the toxins than was previously found. An added complexity is the potential for asexual hybrid endophytes to contain multiple copies of some or all of the genes. While it is possible to make multiple gene “knockouts” using the same selectable marker (Alani et al., 1987; Gorman et al., 1991), the presence of multiple gene copies adds to the technical complexity of a molecular breeding approach. Recombinant DNA approaches to the molecular breeding of endophytes also introduces the “commercial risk” of dealing with a genetically modified organism and the potential for adverse reaction from the general public. One strategy to mitigate this risk would be to cross endophytes of known secondary metabolite genotype and select for progeny with new combinations of those genes. This strategy would be limited, however, to the sexual species, which form either antagonistic or pleiotropic interactions with their host rather than being exclusively mutualistic.

In modifying endophytes, consideration will also have to be given to the biological consequences of those modifications. While biological effects, such as anti-insect activity, have been correlated with the presence of a particular metabolite, it is still unclear whether such effects are the result of the synthesis of a single compound or whether there are synergistic effects. It is also possible that endophyte secondary metabolites may have additional, as yet undescribed, benefits for the symbiosis. It is therefore critical that we better understand the metabolic interaction that occurs between endophyte and host. The molecular cloning of secondary metabolite genes provides us with a key set of tools to better understand that interaction.
8. THE PLANT–ENDOPHYTE SYMBIOSIS

A key molecular tool to investigate the interaction between the grass host and the endophyte is reporter gene technology. The most widely used reporters are gusA (β-glucuronidase, GUS) from *E. coli* (Jefferson et al., 1986) or gfp (green fluorescent protein, GFP) from the jellyfish, *Aequorea victoria* (Chalfie et al., 1994). GUS is a particularly versatile reporter gene system, as there are spectrophotometric, fluorometric, and histochemical assays available to detect enzyme activity (Jefferson, 1987). This allows for both qualitative and quantitative monitoring of fungal gene expression. GUS, under the control of the constitutive *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter has been shown to be particularly useful in monitoring both the distribution and the metabolic activity of endophytes in perennial ryegrass (Murray et al., 1992; Herd et al., 1997).

The major advantage of using GFP as a reporter for monitoring cellular processes is the dynamic nondestructive nature of the assay. Fluorescence can be determined directly, without additional proteins, substrates, or cofactors. GFP has been shown to be a useful reporter of gene expression in several different filamentous fungi, both in culture and during colonization of host tissues (Cormack, 1998; Fernandez-Abalos, 1998; Spellig et al., 1996). The native version of gfp is often poorly expressed in heterologous systems and requires modifications to produce a bright fluorescence. The modified version, sgfp, which has been completely codon-adapted for expression in animal and plant cells, appears to be the most useful form of gfp for monitoring gene expression in filamentous fungi (Chiu et al., 1996). To date there is one report on the use of gfp in *Epichloë* endophytes. *N. lolii* transformants containing the sgfp under the control of the *Aspergillus gpd* promoter fluoresced brightly both in culture and in planta (Mikkelsen et al., 2001).

The cloning of endophyte genes for both primary (isoprenoid, pyrimidine, and vitamin biosynthesis) and secondary (indole-diterpene and ergot alkaloid biosynthesis) metabolism now opens the way to monitor the metabolic interaction between the host and endophyte using both gfp and gus as reporters of the expression of those genes. These constructs will also be useful for determining how both primary and secondary metabolism in the endophyte changes in response to both biotic and abiotic stresses. Other methods that could be used to analyze both qualitative and quantitative differences in gene expression in the symbiosis include cDNA-AFLP (Bachem et al., 1996), suppression subtractive hybridization (Diatchenko et al., 1996), serial analysis of gene expression (Velculescu et al., 1995), and cDNA/oligonucleotide-microarray hybridization analysis (Schena, 1995).

The ability to transform endophytes also opens up the possibility of using endophytes as vehicles for introducing foreign genes into grasses. As
the endophyte is 50–200 times less the biomass of the grass host (Groppe and Boller, 1997; Panaccione et al., 2001), surrogate transformation would be better suited to introducing transgenes that encode enzymes for the synthesis of metabolites that could be systemically transported through the plant, rather than for the synthesis of novel proteins. One such approach has been recently used to introduce the Agrobacterium tumefaciens tzs gene, encoding an isopentenyl transferase, with the aim of developing a novel symbiotum containing an endophyte that synthesizes altered levels of cytokinins (Mikkelsen et al., 2000). Such transformants have the potential to promote host shoot formation and delay leaf senescence. Transformation of the endophyte instead of the grass would have the added biological advantage of excluding transgenes from the pollen.

It is crucial with all of these ex planta modifications that the regenerated endophyte remains compatible with the host. As discussed above, endophytes from perennial ryegrass frequently show morphological instability following isolation from the grass (Christensen et al., 1991). Mutants with a “waxy” phenotype are frequently observed among these morphological variants (Christensen et al., 1993; Scott, 2001). This class of mutants is unable to be reintroduced into the grass host, suggesting that the morphological change that has occurred is associated with a breakdown in compatibility between the endophyte and the grass host. Incompatibility can also occur when endophytes are transferred from their natural host to an alternative host background (Christensen, 1995; Koga et al., 1993). Further work is required to fully understand the mechanisms responsible for host–endophyte incompatibility, but this will be necessary if we are to establish biologically stable novel associations.

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Molecular Aspects of Host–Pathogen Interactions and Ergot Alkaloid Biosynthesis in *Claviceps*

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1. INTRODUCTION

*Claviceps* species, the causal agents of ergot disease, parasitize more than 600 monocotyledonous plants (Bové, 1970), including important crop plants, especially rye and millets, but also wheat, barley, rice, corn, and oats. From an economic point of view, this genus has two faces; the contamination of seed with ergot sclerotia can reduce grain quality severely due to the presence of mycotoxins, the so-called ergot alkaloids, which can have deleterious effects on the central nervous systems of mammalia. On the other hand, these substances have been biotechnologically exploited worldwide on a large scale due to their high pharmacological value. Both aspects ensured continuous scientific interest in this group of fungi in the last 50 years, since both the development of new defense strategies against ergot disease, and strain improvement programs for biotechnological purposes require a detailed understanding of the biology, physiology, and genetics of these fungi. In this review we will try to give an update on research covering both aspects, i.e., research dealing with the interesting phytopathogen *Claviceps*, and investigations on the producers of
biotechnologically interesting substances focusing on genetic and molecular aspects.

The genus *Claviceps* includes more than 30 species, most of them with a defined, narrow host range. Most of the physiological and genetic research in the last years was performed on the species *C. purpurea*, which is exceptional because of its wide host range of more than 400 plant species (Taber, 1985) and the interesting peptide alkaloids that it produces; therefore we will focus here on this species, though references will be made to some other species. We will first give a short introduction to the general pathogenesis of *C. purpurea* and related species, present an update of recent molecular data in this field, and then take a closer look at genetic aspects of ergot alkaloid biotechnology. Due to space limitations, this review can give only an overview and present some highlights (and hence will be subjectively selective); for more detailed information we would like to refer to several recent reviews dealing with specific aspects in a more comprehensive way: Flieger et al., 1997; Keller, 1999; Keller and Tudzynski, 2001; Kozlovsky, 1999; Minghetti and Crespi-Perellino, 1999; Oeser et al., 2001a; Tenberge, 1999; Tudzynski, 1999; Tudzynski et al., 2001.

2. GENERAL PATHOLOGICAL ASPECTS OF THE HOST–PATHOGEN INTERACTION

2.1. Life Cycle

In spring, the biotrophic life of an ergot fungus, e.g., *C. purpurea*, starts with wind-borne ascospores, landing, attaching, and germinating on the pistil surfaces of grass florets at anthesis (Fig. 1). Since all arising stages of the life cycle can develop from one single spore, the ergot fungus is homothallic (Esser and Tudzynski, 1978). Hyphae invade and colonize the ovary, grow down in the direction of the tip of the ovary axis, the rachilla, and establish a specific and persisting host–pathogen frontier. A sphacelial stroma develops in the ovary and produces masses of conidiospores that are exuded into a syrupy fluid (Fig. 1). With this honeydew, the conidiospores are transferred to other florets, spreading the disease spatially in the field (Tulasne, 1853; Engelke, 1902; Kirchhoff, 1929; Langdon and Champs, 1954; Swan and Mantle, 1991). Honeydew production and conidiation cease when the formation of sclerotia starts (Fig. 1). Sclerotia mature in about 5 weeks and typically contain alkaloids. Finally, during autumn, instead of a caryopsis, a ripe sclerotium leaves the spike, therewith making ergot a replacement-tissue disease (Luttrell, 1980). The following spring, germinating sclerotia (Fig. 1) form flask-shaped perithecia and asci therein. After meiosis, needle-like, hyaline, nonseptate ascospores are ejected through the apical pores of asci (Luttrell, 1977) and represent a new primary inoculum.
2.2. Organ Specificity and Host Susceptibility

All ergot fungi infect gynoecia of anemophilous monocotyledons solely. The basis of this strict organ specificity, though not understood, obviously reflects special adaptations. Fungal growth mechanisms are likely adapted to the exceptional molecular architecture of the monocotyledonous cell wall (Carpita and Gibeaut, 1993) and to unique modifications in the pistil tissues (Tenberge et al., 1996a, 1996b; see below). In addition, fungal colonization exhibits some striking similarities to the plant fertilization process, i.e., pollen adhesion and pollen tube growth in the grass pistil. The fungus might mimic pollen tube growth and might use specific components of the signal exchange of the pollen–stigma interaction to grow unrecognized in the ovary. Therefore it might avoid host defense reaction of any type. Host reactions are only very rarely reported (Platford and Bernier, 1970). We recently started a new project to investigate the signaling during the ergot fungus one-way growth.

2.3. Infection Site and Route

Infection of a single host plant is induced by spores landing and germinating anywhere on the pistil surface (Fig. 1) (Engelke, 1902; Kirchhoff, 1929; Campbell, 1958; Luttrell, 1980; Shaw and Mantle, 1980a; Tenberge, 1994; Tenberge and Tudzynski, 1995; Tudzynski et al., 1995; Tenberge et al., 1999). Following penetration in the outer epidermal wall of the pistil, the hyphae keep on growing toward the rachilla. They grow either down the style in the transmitting tissue following the pollen tube path outside the ovule and leaving this way at the micropylar region in direction of the rachilla or, after lateral entrance into the ovary, they grow in the carpel mesophyll toward the ovary base (Luttrell, 1980; Shaw and Mantle, 1980a; Tudzynski et al., 1995). The ovary wall gets completely colonized after about 6 days postinoculation (dpi). Delayed, the ovule is invaded (Kirchhoff, 1929; Campbell, 1958; Willingale and Mantle, 1987). Fungal cells colonize the entire ovary wall, but in the ovarian axis the hyphae stop to spread in the plant tissue. No hyphae emerge beyond the rachilla tip. Thus, a narrow interface between the fungal stroma and the noncolonized host tissue develops, which is finished approximately 6 days after inoculation and persists throughout the remaining life span (Luttrell, 1980; Shaw and Mantle 1980a; Tudzynski et al., 1995; Giesbert et al., 1998). Stoppage of fungal growth in the rachilla has been shown to be most likely caused by host phenolics that accumulate during infection at this site (Mower and Hancock, 1975; Shaw and Mantle, 1980a; Hambrock, 1996) and might inhibit fungal pectin-degrading enzymes, as has been shown in other systems (Mendgen et al., 1996).
FIGURE 1 Life cycle of *C. purpurea*. The different stages depicted are 1, germinating ascospore; 2, a rye floret at anthesis exposing the stigma between the opened glumes; 3, an infected rye ovary during the colonization phase with withered stigma and style, long ovary cap hairs, and the rachilla (asterisk); 4, a sphacelium; 5, a rye ear with honeydew (arrow head) flowing
2.4. Spore Adhesion, Primary Infection, and Colonization

The attachment process of *C. purpurea* spores has not been investigated precisely. On the stigma or style, the stigmatic fluid might offer hydrophilic conditions or, in the case of honeydew-mediated transmittance, the syrpy fluid may support adhesion to the host surface. After attachment, conidia germination starts with the formation of one to several germ tubes (Fig. 1), supported by dew periods, and is accomplished very quickly. Sometimes a limited external mycelium is formed. The faint plant cuticle of the outer epidermal wall is directly penetrated. Infection hyphae originate either directly from the germ tube or from the external mycelium (Fig. 1). Infection hyphae either pass through the outer epidermal cell layer, growing intercellularly into the anticlinal epidermal walls, or hyphae pass through the outer epidermal cell wall away from cellular junctions (Shaw and Mantle, 1980a; Tudzynski et al., 1995; Tenberge et al., 1999). While most ergot fungi penetrate directly without visible changes in morphology of the hyphae involved, some other species develop special morphological structures. *C. gigantea* produces appressoria (Osada Kawasoe, 1986) and *C. fusiformis* bulbous infection structures at the tips of germ tubes (Willingale and Mantle, 1987). After penetration of the cuticle, the mechanism of which is unclear, subcuticular hyphae live within the outer epidermal cell wall. Then the fungi usually grow between epidermal cells into the host apoplast. The thin fungal cell wall and the host cell wall build up an intimate zone of contact while both host and pathogen appear to be healthy (Tudzynski et al., 1995). Before tapping the vascular traces, fungal growth during the colonization phase has been reported to be exclusively intercellular in all ergot fungi investigated (Luttrell, 1977; Luttrell, 1980; Shaw and Mantle, 1980a; Willingale and Mantle, 1987; Frederickson and Mantle, 1988). However, intracellular growth in living host cells has been documented electron microscopically in *C. purpurea* (Tenberge and Tudzynski, 1994; Tudzynski et al., 1995). The interface of the intracellular hyphae, which are completely encapsulated by the host plasma membrane, has developed special adaptations pointing to haustorial functions (Tenberge et al., 1996a; Müller et al., 1997).

out of infected florets; 6, a sphaecial stroma with phialidic conidiophores producing many anamorphous spores; 7, germinating conidiospore on the host ovary cap with subcuticular hyphal growth toward the cellular junction; 8, a mature rye ear with several sclerotia; 9, germinating sclerotium with stromata that differentiate perithecia (☉) in the head periphery containing asci with ascospores.
2.5. **Sphacelial Stromata for Secondary Propagation**

The completion of the persistent frontier includes the active tapping of the xylem as well as the phloem by intracellular hyphae and coincides with the exudation of honeydew, 6–9 dpi, depending on the specific infection conditions (Fig. 1). This indicates the end of the infection stage I (1–5 dpi) and the beginning of the sphacelial phase, stage II, and presents the first macroscopic evidence for infection. The sphacelial stroma (Fig. 1) is evident after 6 dpi and sporulation ceases approximately 11 dpi (Luttrell, 1980). The sphacelial plectenchyma is formed intercalarly (Tulasne, 1853) and accumulates lipids later in this phase. These filamentous hyphae are of the type normally found in axenic culture. At the base of the ovary, proliferation of the fungal cell starts (Kirchhoff, 1929); hyphae accumulate beneath the host cortical layers and break through the epidermis toward the ovarian outer surface (Luttrell, 1980). Finally, fungal cells cause ovary replacement (Willingale and Mantle, 1987). Phialidic conidiophores emerge from the sphacelial stroma possibly favored by hydrophobins (see below). Numerous oblong conidia are produced (Fig. 1). These conidia do not germinate in the honeydew, which is excreted simultaneously, due to high osmotic pressure (Kirchhoff, 1929; Taber, 1985). Some isolates of *C. purpurea* do not produce honeydew; only a few spores were detected later. However, these strains produced normal sclerotia (Mantle, 1967), indicating that the sphacelial phase appears not to be prerequisite for sclerotia development.

2.6. **Ergot Sclerotia**

Within about 5 weeks postinoculation with *C. purpurea* (Kirchhoff, 1929), the maturity of sclerotia is achieved. They are oblong and measure 2–50 mm in length and a few millimeters in diameter. Sclerotia clearly grow epiphytically on top of the ovary stalk and they mostly emerge out of the florets, denoted stage IV, 20 dpi. Therefore, they require protection from desiccation, UV radiation, and mycoparasitism (Parbery, 1996). The hard compact ergot consists of a plectenchymatous, whitish medulla consisting of special storage cells and the outer cortex which gets naturally pigmented dark purple. First, at several places within the sphacelium, sphacelial hyphae differentiate into sclerotial hyphae. Later, the sclerotal plectenchyma develops intercalarly above the stromatic fungal foot by a generative zone as in the sphacelium (Campbell, 1958; Shaw and Mantle, 1980b). Purple pigmentation is the first sign of sclerotial development in *C. purpurea*, about 12 dpi (Shaw and Mantle, 1980b), but the trigger for the change from sphacelial to sclerotial growth is unknown (Parbery, 1996). Changes in cytology of the cells coincide with increasing levels of lipids, which are predominantly triglycerides with the fatty acid ricinoleate (Corbett et al., 1974; Bassett et al., 1972). Lipid content is evident in these storage cells, thus they are packed with osmiophilic globules, in contrast to the differentiating hyphae of the
generative zone. The hyphae which connect the sclerotium to the ovary stalk and form the stable host–pathogen frontier are of neither the sclerotial type nor sphacelial cells. A special function of the fungal foot is also suggested by xylanase protein that has been localized in the sclerotial phase at this host–pathogen interface (Giesbert et al., 1998).

Sclerotia are the only ergot structure containing alkaloids (Ramstad and Gjerstad, 1955), and the pigmentation of the sclerotial cortex might protect these light-sensitive alkaloids (Taber, 1985). In axenic culture, differentiation of the sphacelial-like hyphae into sclerotial-like cells occurs (Kirchhoff, 1929). They accumulate up to 40% of triglyceride/ricinoleate, but cells show no pigmentation and, as in the parasitic state, do not always produce alkaloids (Bassett et al., 1972).

2.7. Assessment of Developmental Strategy

Throughout the ergot-infected pistil, host cells die subsequent to fungal exploitation of living tissue and possibly some due to induced senescence. *C. purpurea* never kills host cells in advance of colonization with the intention to draw nutrition from the killed cells. In addition, due to the unique pathogenesis pattern, the ergot fungi proliferate intercalarly in the ovary basis, which inevitably results in a separation of the host ovary cap. Nevertheless, the separated and colonized tissue stays alive for a while, possibly with nutritional support from the sugary honeydew. Thus, ergot–grass interactions are classified as belonging to the pathosystem free of necrosis in fully susceptible hosts; cell death is not intended but inevitably induced after a while, similar to early host senescence by other biotrophs (Parbery, 1996).

Like other ergot fungi, *C. purpurea* is a true holobiotroph which is an ecologically obligate pathogen and in nature obtains nutrients only from living host tissue while managing to maintain host cell viability for extended periods, and serve as a sink for plant metabolites. *Claviceps* species likely take advantage of the most common source–sink system for synthate in a host by directly tapping the host’s nutrition supply network (Tenberge et al., 1999) and exploiting the plant resources in a working sink (see below). Probably, the fungus has developed specific techniques to maintain the phloem synthate flow (see below). *C. purpurea*, in contrast to many other biotrophs, is able to survive outside living host tissue while growing saprophytically in axenic culture.

3. MOLECULAR ASPECTS OF PATHOGENICITY

The molecular genetics of the interaction of *C. purpurea* with its major host plant *S. cereale* has been studied in detail in the last years, using two complementing approaches to find factors which are essential for pathogenicity and virulence:
a direct approach focusing on specific aspects of the interaction (e.g., cell wall-degrading enzymes, cell wall components, active oxygen species detoxification, etc.), including functional analyses of genes probably involved in these specific aspects; and a more general approach, based on an EST (expressed sequence tags) library of *C. purpurea*-infected rye ovaries, i.e., an unbiased study of the fungal genes expressed *in planta*, with the chance to identify new, so far unconsidered factors. In this chapter we will first discuss the various specific aspects of the interaction which have been investigated in the last years, and we will present data on the recently established EST library.

### 3.1. Cell Wall-Degrading Enzymes (CWDE)

The inter- and intracellular growth in the cell wall apoplast of the histologically heterogeneous ovary is an important characteristic of *C. purpurea*. The splitting of middle lamella zones and direct penetration of host cell walls in numerous different grasses prove that *C. purpurea* is well adapted to the monocotyledinous cell wall habitat, and indicate that the pathogen uses secrutable CWDE in a well-controlled manner. Since grasses have developed a special cell wall type containing low amounts of pectins and considerably high amounts of glucurono-arabino-xylans (GAX) in addition to the major polysaccharide portion of cellulose (Carpita and Gibeaut, 1993), especially xylanases and cellulases, but also pectinases are expected to be necessary for breaking down the major cell wall components during infection. In addition to being necessary for growth within the tissue, this breakdown of cell walls can also be important for nutrition during colonization of the ovary. This idea is supported by stimulation of growth in culture by cell wall extracts of ovaries (Garay, 1956).

The molecular architecture of the host–pathogen interface has been studied in detail in this system, with emphasis on interaction specific reactions, e.g., polymer alterations and protein secretion, at the electron microscopical level. This molecular cytological study is complemented with a molecular genetic approach in a concerted effort to elucidate the role of CWDE in ergot pathogenicity. In the following a short update is presented of the information available for the major CWDE classes.

#### 3.1.1. Pectinases

Pectolytic enzymes had been found in cultures of *C. purpurea*, in honedew and in infected tissue extracts, by Shaw and Mantle (1980a). Immunogold labeling with the monoclonal antibodies JIM5 and JIM7 showed that—in contrast to the model-based expectations—the two major pectin types, non-methyl-esterified and methyl-esterified galacturonan, are simultaneously present in considerable amounts in the cell walls along the usual infection path in healthy carpels (Tenberge et al., 1996). During infection of rye, a local molecular pectin
modification as well as degradation have been demonstrated for the host cell wall and the middle lamella zone at the interface of subcuticularly and intercellularly growing hyphae in situ. Chemical demethylation and immunogold labeling showed a high local content of galacturonan that was completely absent in late infection phases, providing evidence for the secretion of pectinolytic enzymes in planta. The cellular junctions were shown to contain a high percentage of unesterified pectin. Therefore, polygalacturonase activity could be important for epidermis penetration and entry into the middle lamella from the intercellular spaces, which is not continuous along the infection route.

Using a heterologous gene from *Aspergillus niger* as probe, two putative endo-PG genes were cloned and characterized (Tenberge et al., 1996); they are closely linked in a head-to-tail arrangement and show 95% identity, pointing to a recent gene duplication event. By reverse transcriptase polymerase chain reaction (RT-PCR) it could be shown that both genes are expressed throughout the first 3 weeks of infection, i.e., during the colonization phase and the early sclerotium development. The special head-to-tail arrangement allowed a one-step gene inactivation of both genes by a replacement approach. The analysis of two independent double-mutant strains showed a drastic effect: the mutant strains are significantly impaired in virulence (the infected ovaries produce no honeydew and no sclerotia), indicating an essential role of pectinolysis for pathogenicity (Oeser et al., 2002a).

### 3.1.2. Cellulases

*C. purpurea* can penetrate plant cell walls directly; at the interface of intracellular hyphae, the host cell wall obviously is lacking (Tudzynski et al., 1995). With the use of specific enzyme-gold probe, a lack of β-1,4-glucan in host cell walls has been found at this site and additionally at host–pathogen interfaces of intercellular hyphae, pointing to the enzymatic action of cellulases in ergot infection (Müller et al., 1997). However, cellulolytic activity could never be detected in liquid culture, only on solid medium using substrate staining (Müller, 1997), indicating a strict regulation of cellulase activity. So far, one gene has been cloned which probably is involved in cellulose degradation (Müller et al., 1997): *cpcel1* probably encodes a cellobiohydrolase (lacking the substrate-binding domain). The gene was shown by RT-PCR to be induced during the first days of infection of rye. Therefore, this cellobiohydrolase may be involved in the penetration and degradation of host cell walls. However, deletion of the gene by transformation with a replacement vector showed no effect on pathogenicity (U. Müller and P. Tudzynski, unpublished data). Since the presence of (an) additional cellulase gene(s) in *C. purpurea* cannot be excluded, the role of these enzymes in the interaction is still open.
3.1.3. Xylanases

\( \beta \)-1,4-Xylan has been localized in rye ovary cell walls throughout the infection route by the enzyme-gold technique (Giesbert et al., 1998), confirming that this major cell wall component of grass leaves is also a structural compound in ovary cell walls. The \( \beta \)-1,4-xylan is expected to represent only the backbone of GAX. Arabinofuranosyl epitopes, one of the possible side chains in GAX, were also localized in ovary cell walls (Giesbert et al., 1998). Absence of xylan in late infection stages and xylan alteration early in infection was visualized by transmission electron microscopy (TEM) and after silver enhancement in LM (Heidrich and Tenberge, 2000), strongly suggesting the secretion of xylanolytic activity by the fungus. In fact, xylanase activity could be detected in axenic culture, and the secretion of ergot xylanases during infection of rye has been demonstrated in situ using three different heterologous antibodies in tissue printing experiments (Giesbert et al., 1998).

So far, two putative endo-\( \beta \)-1,4-xylanase genes have been cloned and characterized in \textit{C. purpurea}: \textit{cpxyl1} and \textit{cpxyl2}, probably coding for family G and family F enzymes, respectively (Giesbert et al., 1998). Both genes are expressed \textit{in planta} throughout the whole infection period as shown by RT-PCR. Using a gene replacement approach, single mutants (for both genes) and double mutants were obtained; they showed a significant reduction in total xylanase activity in axenic culture. Gel electrophoresis and activity staining showed loss of corresponding xylanase activity bands, confirming that \textit{cpxyl1} and \textit{cpxyl2} indeed encode xylanases. The effect of the deletions on pathogenicity was not nearly as pronounced as with the PG mutants, but at least the double mutants seem to show a slightly retarded development \textit{in planta} (Giesbert et al., 1998; J. Scheffer, A. Fleissner, P. M. Heidrich, B. Oeser, K. B. Tenberge, and P. Tudzynski, unpublished data).

3.1.4. \( \beta \)-1,3-Glucanase

Plant phloem exudates are the main nutritional source of \textit{C. purpurea} (Mower and Hancock, 1975). To exploit this natural sink, the fungus secretes several enzymes and the persisting host–pathogen frontier is developed structurally for attaching and absorbing (Luttrell, 1980). In contrast to uninfected ovaries, phloem callose is not present in infected ovaries or is at least distinctly reduced (Tudzynski et al., 1995). This unblocking of sieve elements may be the reason for honeydew exudation, due to increased flow of assimilates to the infected floret. The current opinion of the mechanisms is that ergot fungi enzymatically degrade the phloem callose by secreting \( \beta \)-1,3-glucanases, which have been purified from axenic cultures of \textit{C. purpurea} (Brockmann et al., 1992). This enzyme has been localized throughout the colonization phase and in structural elements of the fungal secretion pathway by immunogold labeling, proving the fungal origin of
the β-1,3-glucanase activity found in infected ovaries and honedew (Tenberge et al., 1999). Immunogold electron microscopy also documented that the secreted enzyme is diffusing into the host apoplast. The enzyme was shown to reach the typical deposition sites of callose, indicating an enzymatic suppression of putative plant defense reactions. The host phloem was colonized inter- and intracellularly. Hyphae penetrated into the pectic middle lamella of sieve plates and intense immunolabeling for β-1,3-glucanase in this area supports the “phloem unblocking” hypothesis. However, the fungus might influence the host phloem unloading by other techniques.

Recently, a putative mixed-link (β-1,3/1,4)-glucanase gene was identified within a EST library of in planta-expressed genes of C. purpurea (see below), perhaps opening the way for a functional analysis also of this enzyme system.

Taken together, these data indicate that CWDE (especially pectinolytic enzymes) play an important role in the colonization of rye ovarian tissue by C. purpurea.

3.2. Enzymes Involved in the Generation and Scavenging of Active Oxygen Species (AOS)

One of the earliest defense reactions of plants against pathogens is the transient formation of active oxygen species (AOS: O₂⁻, H₂O₂, OH⁻) termed oxidative burst in analogy to mammalian systems (Lamb and Dixon, 1997). A well-established role of AOS in early defense against pathogens is the induction of rapid reinforcement of the host cell wall by cross-linking of cell wall proteins. Additional (but not generally accepted) functions of AOS are the induction of the hypersensitive response (HR), triggering rapid necrosis at the infection site, and activation of late defense genes in the surrounding tissue.

The oxidative-burst-derived AOS most probably has direct impact on the pathogen, causing, e.g., membrane damage and inactivation of proteins. All living cells have developed protective systems against oxidative damage, including nonenzymic AOS scavenging mechanisms such as the accumulation of ascorbic acid, mannitol, GSH, etc., and enzymes which rapidly detoxify AOS. The latter include superoxide dismutases, which dismutate O₂⁻ to H₂O₂, and catalases, which decompose H₂O₂ into water and O₂, and peroxidases, which can use a variety of substrates for the reduction of H₂O₂ to H₂O. Bacterial pathogens of mammals have highly efficient enzymatic AOS-scavenging systems: in Staphylococcus aureus, e.g., secreted catalase activity seems to be correlated with its survival of phagocytosis as well as with the mortality levels of infected mice (Mandell, 1975). Periplasmic Cu,Zn-superoxide dismutases obviously are involved in survival strategy of Salmonella typhimurium (De Groote et al., 1997).

Comparable analyses are rather limited in pathogenic fungi–plant interactions. Although AOS-scavenging systems have been implicated in the
pathogenesis of Aspergillus fumigatus and Cryptococcus neoformans (Hamilton and Holdom, 1999, Jacobson et al., 1994), it has yet to be determined if they represent significant virulence factors in these systems. We have recently initiated a detailed study on the role of AOS and AOS-scavenging systems in the interaction of Claviceps purpurea and rye. Though in this biotrophic system no strictly incompatible reactions (and hence no HR) are known, the involvement of AOS in the interaction is implicated by several observations (Tenberge, 1999).

Since there is evidence for the presence of H$_2$O$_2$ in rye tissue colonized by C. purpurea, we first focused on H$_2$O$_2$-decomposing enzyme systems. Detailed cytological and biochemical analyses by Garre et al. (1998a) showed that C. purpurea produces four different catalases in axenic culture, CAT A–D. Whereas CAT A is an intracellular form, CAT B seems to be mainly cell wall-associated, and CAT C/D are secreted forms. The catalase isoforms CAT A, B, and D were also detected in planta, CAT D even in honeydew of infected plants. This represents (to our knowledge) the first example of a secreted catalase in a phytopathogenic fungus. CAT C/D were shown to be encoded by one gene, $cpcat1$, as disruption of this gene resulted in loss of both isoforms, in axenic culture and in planta (Garre et al., 1998b). RT-PCR analysis showed that the gene is expressed in all stages of infection. However, $cpcat1$ deletion mutants were not impaired in pathogenicity, demonstrating that this major extracellular catalase is not essential, at least not on rye and under the (near natural but still artificial!) inoculation conditions tested. Since with CAT B there is still a probable cell wall-associated catalase present, which could take over the protection against H$_2$O$_2$ in the mutants (though no increased activity of CAT B in the mutants could be detected), a second putative catalase gene, $cpcat2$, localized in the ergot alkaloid biosynthesis cluster (see below) was cloned and disrupted, again without significant effect on virulence (S. Moore and P. Tudzynski, unpublished data). Since the catalase system in C. purpurea obviously is complex (recently an additional catalase isoform, CAT E, was detected in axenic culture; S. Moore and P. Tudzynski unpublished data), presently conclusions about the importance of these enzymes for the Claviceps–rye interaction would be premature.

Recently, a probably cell wall-associated SOD was detected by IEF/activity staining experiments (Moore et al., 2002). Using a PCR approach based on degenerated primers derived from SOD sequences from various organisms, a gene ($cpsod1$), showing significant homology to Cu-Zn-SODs was cloned and demonstrated expression throughout all phases of infection. Deletion of $cpsod1$ led to disappearance of the major secreted SOD form, although the gene contains no apparent signal peptide. The deletion mutant showed some limited reduction of parasitic properties: the appearance of honeydew in infection tests was retarded for 1 day and the amount of honeydew was reduced (Moore et al., 2002); this retardation is probably linked to a slightly reduced growth in axenic culture. It is questionable if this minor defect has any practical
implications in nature. It shows, however, that SOD1 activity is not really essential for pathogenicity in the laboratory test system.

To test if the combined loss of both major extracellular AOS scavenging activities has impact on pathogenicity, cpcat1/cpsod1 double mutants were created (S. Joshi, P. Tudzynski, unpublished). In these mutants the minor effect observed for Δcpsod1 was slightly more pronounced, i.e., the delay in honeydew production was clearly 1 day, and the amount of honeydew was significantly reduced. However, in spite of this retardation, normal sclerotia are formed, i.e., the infection is successful.

Obviously, the defense system against AOS is highly complex in C. purpurea, as in other eukaryotic systems. Therefore we initiated a more general approach to identify genes induced under oxidative stress. Growth on medium with a high copper content was chosen for the induction of oxidative stress. Copper acts as a Fenton catalyst in the generation of AOS (Stohs and Bagchi, 1995), and induces transcription of Cu-Zn-SOD and catalase in yeast (Carri et al., 1991; Gralla et al., 1991; Lapinskas et al., 1993). A cDNA library of RNA from copper-induced mycelium of C. purpurea strain 20.1 was established and differentially screened with copper-induced and copper-starved cDNA (Oeser et al., 2002b). In a first round of experiments 40 differentially hybridizing c-DNA clones were identified. Northern analysis showed that several of them were also induced by H₂O₂ in axenic culture, confirming that the copper-stress approach was successful for the identification of genes induced by oxidative stress. A complete list of the annotated clones is presented by Oeser et al. (2002b); there are several interesting clones, e.g., for a high-affinity Fe-transport system and a hydrophobin (see above); the detailed functional analysis of these clones is under way. Of special interest also are “orphan” clones that show no significant homology to any database entry and could represent C. purpurea-specific genes.

Moreover, another new and fascinating aspect of AOS-related gene expression in C. purpurea came from the EST data analysis (see below); one of the potential transcription factors identified in the in planta expression analysis shows homology to an oxidative stress-related factor in mammalian cells (atf1). This gene (cptf1) was shown to be induced in axenic culture of C. purpurea by H₂O₂. The corresponding gene has been cloned and characterized (Joshi et al., 2003); it is expressed in all stages of infection. A targeted inactivation of cptf1 showed that CPTF1 controls catalase activities in axenic culture; the mutants have reduced virulence. By this H₂O₂-induced transcription factor, and if its inactivation has an impact on pathogenicity. This could allow a more general approach to the understanding of the impact of AOS stress defense in the development of C. purpurea in planta.
3.3. **Hydrophobins**

Hydrophobins, a class of small hydrophobic proteins which have been detected in several filamentous fungi (Wessels, 1994), are characterized by their ability to form amphipathic layers at hydrophobic/hydrophilic interphases. They are abundant in aerial structures of fungi, such as aerial hyphae, conidia, and fruiting bodies. They are implicated in the interaction of phytopathogenic fungi and their hosts, e.g., in *Magnaporthe grisea* and *Cryphonectria parasitica* (for review, see Kershaw and Talbot, 1998). In a (nonpathogenic) strain of *Claviceps fusiformis* used for submersed production of ergot alkaloids, a gene (*cfth1*) was detected encoding a new type of hydrophobin: it contains three hydrophobin domains separated by asparagine/glycine repeats (Arntz and Tudzynski, 1997). Detailed biochemical analysis resulted in the identification of the gene product, a full-sized protein (i.e., not processed down to single hydrophobin units) showing properties of a typical class II hydrophobin (de Vries et al., 1999).

Recently, a comparable gene, *cpph1*, was identified in *Claviceps purpurea*, which codes for a protein with an even higher complexity, a pentahydrophobin (Mey et al., 2003). It consists of five units showing significant homology to class II hydrophobins, interrupted by GN-repeat regions, which could form amphipathic α-helices; the amino terminus contains a glycine-rich region which could be involved in attaching the protein to the cell wall. The structure of *cpph1* is comparable to that of *cfth1* of *C. fusiformis*; the presence of long direct repeats within *cpph1* and the high homology of the internal three modules suggest a recent generation of this gene from a tripartite precursor. By in-situ hybridization it could be shown that *cpph1* is expressed *in planta*, especially in two areas: in external and penetrating hyphae and in conidiogenic hyphae (Tenberge et al., 1998), indicating that this special protein could be involved in establishment of fungus–host contact and coating of spores.

Using a gene-replacement approach, two deletion mutants of *cpph1* could be obtained; detailed pathogenicity tests showed that these mutants are not impaired in their pathogenicity. Thus also this uniquely structured gene expressed in a tissue-specific manner does not seem to be essential for the development of disease symptoms (on rye!).

Within an EST library of *C. purpurea* (see below), a gene encoding a “classical” (single-unit) class II hydrophobin (*cph1*) has been detected; the gene is expressed in most stages of infection and is induced by copper (see above), but a specific role has not yet been assigned to it.

3.4. **Signal Chain Components**

Pathogenic fungi, even more than other organisms, rely on their ability to respond to signals from the environment. These signals are transduced from the cell surface to the target genes, resulting in altered gene expression in response...
to changes in the environment. Recent studies have demonstrated that phytopathogenic fungi have developed specific signal transduction pathways for pathogenesis. Several genes coding for components of signal chains have been characterized from phytopathogens and functionally analyzed, e.g., for subunits of heterotrimeric GTP-binding proteins, various protein kinases, and other components of the cAMP transduction pathway (for review, see Tudzynski and Tudzynski, 2001). Since especially compounds of the MAP-kinase cascade were shown to have an impact on pathogenicity in various fungi (Xu, 2000), we initiated an analysis of this signaling cascade in *C. purpurea*. Using a degenerated primer approach, two putative MAP kinase genes were cloned and characterized (Mey et al., 2002a; Mey et al., 2002b): *cpmk1* and *cpmk2*, showing significant homology to the MAP kinase genes *mpk1* and *mps1* of *M. grisea*, respectively (Xu and Hamer, 1996; Xu et al., 1998). Both genes are expressed *in planta* throughout the whole colonization phase. Since both corresponding *M. grisea* genes have been shown to be essential for pathogenicity, though with different specific effects, we chose two approaches to study the function of these homologs in *C. purpurea*: (1) knockout mutants were obtained by a gene-replacement approach, in which the deletion mutants are completely apathogenic; (2) complementation of the corresponding *M. grisea* deletion mutants, to see if the signal transduction pathway in these divergent fungi are comparable, in which both *C. purpurea* genes fully complement the corresponding *M. grisea* mutant, under control of their own promoters (Mey et al., 2002a; Mey et al., 2002b).

One fascinating aspect of signaling mutants which are affected in their pathogenicity is that they present invaluable tools to study the very first parts of the respective signal chain (i.e., the receptors) and to identify target genes, which together obviously make up the pathogenicity phenotype. This raises the analyses from a “hit-and-run” strategy to a more global understanding of the interaction system. In *C. purpurea* it will be especially interesting to learn more about the strict organ specificity, and about the mechanisms which guide the hyphae along the path of the pollen tube down to the vascular system.

### 3.5. Genomics Approach: In Planta-Expressed Genes

In order to obtain a deeper insight into the genes/factors involved in development of *C. purpurea* on rye, we initiated an unbiased analysis of *in planta* expressed genes of the fungus. Rye plants were inoculated by pipetting conidia suspension into the floral cavities at anthesis in an infection test that was very near natural conditions. At 5, 10, 15, and 20 dpi (corresponding to stages I–IV, see *Sec. 2*), infected ovaries were harvested and immediately lyophilized. RNA was prepared and used for cDNA synthesis. Mixed RNA aliquots of all four stages were used
for the establishment of a cDNA library (in ZAPII). The resulting cDNA clones were sequenced (from both sides), yielding the first (small-scale) EST library of *C. purpurea*. A BLAST analysis of all sequences resulted in annotation of only 33% (i.e., these clones showed significant homology to genes with known function); about 13% were homologous to other database entries without apparent function; and more than 50% were “orphans” without any comparable sequence in the database. The percentages of these three categories are similar to the values reported for other EST banks (e.g., *Neurospora crassa*, Nelson et al., 1997; *Mycosphaerella graminicola*, Keon et al., 2000). A complete list of the annotations available so far is given in Oeser et al. (2002b); there are several interesting candidate genes that could represent pathogenicity factors, but the detailed functional investigation will need some time. Also here—as with the Cu-induced genes, see above—the “orphan” clones might be especially interesting.

An interesting aspect of the EST analysis is to use the clones for establishment of a transcription profile. To link the cDNA clones to a specific stage, all cDNA clones were dot-blotted and separately hybridized with cDNA probes derived from infection stage I (about 5 dpi; few visible symptoms), II (about 10 dpi; honeydew and conidia production; sphacelium), III (about 15 dpi; sclerotial development starting; sphacelium > sclerotium), and IV (about 20 dpi; progressed sclerotial development). Hybridization signals have been obtained for only 58 clones so far, probably due to the limits of the dot-blot procedure. Details are presented by Oeser et al. (2002b).

Expression of several genes could be linked to the early stages of infection, which probably is the most crucial period determining the “success” of infection; others are expressed in the switch from sphacelial to sclerotial growth; and others are obviously expressed throughout the whole infection cycle. Apart from several “orphan” clones within these different groups, expression of some annotated genes can be linked to specific phases, e.g., for the initial phase a “mixed-link” β-glucanase (which could correspond to the β-1,3-glucanase described in *C. purpurea*, see above), and a homolog of the “la costa” gene of *Drosophila melanogaster* (involved in larval segment polarity). Thus, though still in its infancy, the detailed analysis of *in planta*-expressed genes already shows the great potential of this approach; it will yield new and nonanticipated genes that play a role in the interaction. Together with the still valid direct/specific approach outlined above, molecular genetic analysis will allow to come to a full understanding of the complex mechanisms underlying pathogenesis of *C. purpurea*. 

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4. GENETICS OF ERGOT ALKALOID BIOSYNTHESIS

4.1. Biochemistry and Pharmacology

Ergot alkaloids are mycotoxins produced (mainly) by Claviceps species which have deleterious effects on the central nervous system of mammalia; on the other hand, they have a long biotechnological tradition with manifold applications in therapy of human central nervous system (CNS) disorders (for reviews, see Keller and Tudzynski, 2001; Tudzynski et al., 2001). Chemically they are 3,4-substituted indole derivatives having a tetracyclic ergoline ring structure (Fig. 2).

![Ergot alkaloids schematic structure](image)

**Figure 2** Ergot alkaloids: schematic structure of the ergoline ring system, of D-lysergic acid amides, and of agroclavine as an example for the “simple” clavine alkaloids. (Modified from Tudzynski et al., 2001.)
The biosynthetic pathway of these secondary metabolites has been investigated in detail (for review, see Floss, 1976; Keller, 1999; Tudzynski et al., 2001), though only a few of the enzymes involved have been characterized; as shown in Fig. 3 for ergotamin, the specific pathway starts with the formation of dimethylallyl-tryptophan, proceeds via several oxidation steps and ring modifications to lysergic acid, and finally a small peptide moiety is attached in amide linkage via a carboxy group in the 8-position (Fig. 3B). The various Claviceps species differ in their ability to produce specific alkaloids: C. purpurea strains normally can produce lysergic acid derivatives, mainly ergopeptides, whereas C. paspali and C. fusiformis produce only the simple clavine alkaloids (e.g., agroclavine).

In field isolates of C. purpurea, the alkaloids are only produced in planta, in sclerotial tissue. In the past, contamination of cereals with sclerotia has led to severe disease symptoms in humans, known as St. Anthony’s fire in the Middle Ages. In modern mills, cereals are mechanically cleaned from sclerotia, which due to a growing number of grain-sized sclerotia gives no complete protection. Contamination of flour with ergot alkaloids is still a problem, and has even increased in the last years, e.g., due to C. purpurea-susceptible new hybrid rye varieties.

The therapeutic potential of ergot alkaloids was recognized already in the Middle Ages, e.g., in childbirth or abortion (hence the common name “mothercorn”). Intensive analyses of the purified alkaloids in the last 50 years have clarified their various toxic and pharmacological effects, which are caused by the structural homology of their tetracyclic ring system to neurotransmitters such as noradrenaline, dopamine, and serotonin (for review, see, e.g., Berde and Stürmer, 1978).

Biotechnological explanation of ergot alkaloids in the last decades involved improvement of production strains with respect to yield and purity of products. In parallel, however, considerable efforts were made to improve ergot alkaloids as therapeutic agents by chemical modifications of natural ergolines, especially to narrow the specificity of compounds. Because of rapid progress in molecular genetics of C. purpurea, “metabolic design” of this pathway, including completely new products, is imaginable. Here we will focus on the present state of the genetics of the ergot alkaloid biosynthesis pathway.

### 4.2. Classical Genetics

Most field isolates of C. purpurea obtained in screening programs over the years yielded interesting strains producing different, specific alkaloids (or desired mixtures) at moderate rates in planta only, thus they were not suited for submerged production of alkaloids. Strain-improvement programs focused therefore on an increased capacity of strains to produce high amounts of a specific alkaloid in axenic culture. This was mainly achieved, as in other
FIGURE 3 Ergot alkaloid pathway in *C. purpurea*. (A) Ergoline ring biosynthesis up to lysergic acid. (B) Ergopeptide biosynthesis (ergotamin) starting from lysergic acid. (Modified from Tudzynski et al., 1998.)
strain-improvement programs of secondary metabolite-producing fungi, by a stepwise mutagenesis selection process.

Mutagenesis as a means to improve the selection procedures for getting alkaloid high producers has been broadly applied in many laboratories (Didek-Brumec et al., 1987; Kobel and Sanglier, 1978). Uninucleate conidia have been treated with various mutagens such as radiation (UV, X-ray) or chemicals (nitrous acid, N-nitrosoguanidine, nitrosourea, ethylmethanesulfonate, ethyleneimine) and the resulting colonies tested for improved alkaloid production in liquid and solid media (Strnadová, 1964; Kobel and Sanglier, 1973; Srikrai and Robbers, 1983; Didek-Brumec et al., 1987). In the case of strains unable to form conidiospores, protoplasts have been used as unicellular units for mutagenesis (Spalla and Marnati, 1978). Protoplasting of hyphae previously grown in the presence of a mutagen such as MNNG or EMS followed by regeneration to single-cell-based colonies is another method to get mutants with improved capacity to synthesize ergot alkaloids (Keller, 1983).

Though \emph{C. purpurea} is a perfect fungus, sexual genetics cannot be used on a broad scale for strain improvement, only for parasitic production strains (see Tudzynski, 1999). Most submerged-producing mutant strains are imperfect (and often lose the ability to sporulate). However, most \emph{C. purpurea} field isolates are highly heterokaryotic, and heterokaryosis can be easily induced in laboratory strains by protoplast fusion or anastomoses; therefore parasexual genetics offered an alternative strategy for strain improvement. The importance of heterokaryosis for the production of alkaloids is controversially discussed in the literature: Esser and Tudzynski (1978) could demonstrate that homokaryotic mycelia can produce alkaloids, in contrast to earlier observations (e.g., Amici et al., 1967) that only heterokaryotic strains were good producers. It could be shown by several groups that formation of heterokaryons between strains of different genetic background had indeed significant impact on alkaloid biosynthesis; here probably effects such as heterosis, complementation, gene dosage, etc., are important. Spalla et al. (1976) showed that a forced heterokaryon between \emph{C. purpurea} strains producing ergochristine and ergocornine/ergocryptine, respectively, produced all three alkaloids. Also, protoplast-mediated fusion between species have been used successfully to obtain different spectra of alkaloids, e.g., between an ergotamin-producing \emph{C. purpurea} strain and a clavin-producing \emph{C. fusicomis} strain (Robbers, 1984; Nagy et al., 1994) and strains of \emph{C. purpurea} and \emph{C. papsali} (Spalla and Marnati, 1981). Therefore, fusion of strains and generation of defined heterokaryons represent a powerful tool for improvement even of imperfect submerged-production strains.
4.3. Molecular Genetic Approaches

Schardl and co-workers were the first to clone a gene of the alkaloid biosynthetic pathway (Tsai et al., 1995): \textit{dmaW} was shown to encode dimethylallyltryptophan synthase (DMATS), the first enzyme of the specific part of the pathway (see Fig. 3). A cDNA clone of the same gene was obtained during a differential cDNA screening approach (Arntz and Tudzynski, 1997), using a cDNA library from an alkaloid-producing culture of strain ATCC 26245 (the same strain as used by Tsai et al., 1995) and cDNA preparations from an alkaloid-producing and nonproducing culture as probes. Northern analysis confirmed that the gene was induced concomitant with ergot alkaloid production, the first evidence for transcriptional control of alkaloid biosynthesis.

The strain ATCC 26245 used in these first molecular analyses of the ergot alkaloid pathway turned out to be a \textit{C. fusiformis} isolate (Pazˇoutová and Tudzynski, 1999). Since this strain produces no peptide alkaloids, i.e., it probably lacks the final part of the pathway, it is not optimal for the molecular analysis of the alkaloid pathway. Therefore, a detailed analysis of genes involved in the ergot alkaloid biosynthesis (Tudzynski et al., 1999) used a confirmed \textit{C. purpurea} strain (P1), a derivative of strain 1029 (obtained after two rounds of mutagenesis; Keller, 1983), which produces peptide alkaloids in axenic culture (mainly ergotamin). Two putative DMATS genes were cloned from strain P1, one of which obviously represents an inactive copy (containing a frame shift mutation due to an internal 7-bp duplication; Arntz, 1999). The derived amino acid sequence of the active copy, termed \textit{cpd1}, showed about 70% homology to the corresponding gene of strain ATCC 26248, confirming the distant relationship of these two strains. Recently this analysis of \textit{dmaW} homologous genes was extended to several isolates of \textit{C. purpurea} and a related endophytic member of the Clavicipitales, \textit{Balansia obtecta}. All \textit{C. purpurea} isolates tested contained two \textit{dmaW}-like gene copies, which obviously constitute two separate developmental lines, of which only one seems to be involved in ergot alkaloid biosynthesis. \textit{B. obtecta} contained only one DMATS gene (Arntz, 1999; B. Wang, T. Correia, P. Tudzynski, and C. Schardl, unpublished data).

A chromosome-walking approach starting from \textit{cpd1} led to the detection of a putative ergot alkaloid gene cluster in this strain: a gene for a putative trimodular peptide synthetase (cpps1) was located closely to \textit{cpd1}. Internal peptides obtained by Keller and co-workers from the lysergylpeptide synthetase 1 (LPS1) from \textit{C. purpurea} (Riederer et al., 1996; U. Keller, unpublished data) matched exactly to parts of the gene sequence. Therefore, \textit{cpps1} obviously encodes this enzyme, which catalyzes the activation of the three amino acids of the peptide part of ergotamin and links them to the activated lysergic acid (see above). Close neighbors of the genes \textit{cpd1} and \textit{cpps1} for the first and one of the final steps of the detected \textit{cpox1}, a good candidate for the chanoclavine cyclase,
and cpox2, a putative dehydrogenase, as well as several yet unidentified open reading frames (Tudzynski et al., 1999).

Extensive sequencing of the genomic region “left” of the initially found part of the ergot alkaloid gene cluster (T. Correia, Y. Lübbe, and P. Tudzynski, unpublished data) led to the identification of several new candidates for alkaloid biosynthesis genes. These include two putative monomodular peptide synthetase genes (cpps2, 3), one of which could code for the lysergic acid-activating enzyme (LPS2, Riederer et al., 1996; see above); one potential P450-monoxygenase (cp450-1, 2), which could be involved in the last steps of lysergic acid biosynthesis or the final step of ergopeptin biosynthesis (see Fig. 2); and several oxidases (cpox1, 2, 3) which could have functions in the early steps of biosynthesis. At the left border of the so far available sequence a putative “housekeeping” gene encoding an enzyme of the amino acid biosynthesis (isopropylmalate-dehydratase) indicates the “left end” of the cluster.

The idea that all these genes might indeed be part of an alkaloid cluster was recently substantiated by expression studies: Northern analysis showed that all these genes (also the peptide synthetases) are induced in alkaloid-producing cultures of strain P1 (low phosphate) and repressed under high-phosphate conditions (Y. Lübbe, T. Correia, and P. Tudzynski, unpublished data). However, gene disruption studies and heterologous expression in *Escherichia coli* yeast will be necessary to confirm specific functions of these genes in ergot alkaloid biosynthesis.

5. PERSPECTIVES

The data presented in this review show that in the major fields of research on *Claviceps*, both phytopathology and biotechnology, the molecular genetic approach has opened fascinating new perspectives. *C. purpurea* is an interesting model system for molecular phytopathology, as a biotrophic, organ-specific pathogen, combining the fascinating characteristics of a highly evolved interaction system from a long co-evolutionary process with a comparably easy experimental access. Molecular techniques such a targeted gene inactivation and in-situ gene expression studies have for the first time allowed the unequivocal identification of specific pathogenicity/virulence factors. Moreover, recent developments such as analyses of signal chains and transcription factors, as well as the application of “genomics,” now open the way for a detailed understanding of the complex system as a whole, though we have to admit that presently the major outcome is merely a realization of the high degree of complexity involved!

From a biotechnological point of view, the impact of molecular genetics lies mainly in the detailed analysis of the biosynthetic pathway and in the option to create new alkaloids with higher pharmacological value, especially those showing a new or more specific mode of action. This is especially interesting
since the importance of the “classical” ergot alkaloids is decreasing, mainly due to the undesired side effects.

In both areas of research the scientific community can expect major contributions in the next years; scientific interest in the genus Claviceps and especially C. purpurea will certainly be catalyzed by these new developments.

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Ergot Alkaloid Toxicity

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1. INTRODUCTION
During the Middle Ages, there were numerous incidences of human toxicosis involving hallucinations, convulsions, delusions, and gangrene. The syndrome became known as “St. Anthony’s fire.” In some instances, people actually jumped from buildings during hallucinogenic states. Barger (1931) determined that ergot alkaloids from the *Claviceps purpurea* fungus were the causative agent. Ergot alkaloids are characterized by an indole group which is a component of a tetracyclic ring known as an ergoline ring (Lorenz, 1979). Most of the sclerotia, containing the ergot bodies, can be removed during the grain cleaning process. However, small amounts have been detected in a variety of grains destined for human consumption (Scott et al., 1992). Therefore, even with modern grain processing methods, ergot alkaloids pose some threat to the human population.

2. INTERACTION OF ERGOT ALKALOIDS WITH RECEPTORS
Ergot alkaloids exhibit a high affinity for α-adrenoreceptors, D₂ dopamine receptors, and several subtypes of 5-HT receptors (Pertz and Eich, 1999). The various alkaloids may act as receptor agonists or antagonists, depending on the receptor and the particular alkaloid. Ergovaline was shown to activate 5-HT₂A
receptors and thus induce contractions of blood vessels (Dyer, 1993). Thus, blood flow to the vascular beds was reduced. Further research by Schoning et al. (2001) confirmed the interaction of ergovaline with 5-HT$_{2A}$, 5-HT$_{1B/1D}$, and $\alpha$-adrenoreceptors. Many of the toxic effects of ergot alkaloids can be explained by changes in vascular blood flow as proposed by Cross (1997). Strickland et al. (1994) reported that ergovaline was a D$_2$ dopamine receptor agonist in rat pituitary cells. In this study, ergovaline blocked the release of prolactin by cultured pituitary cells. The D$_2$ dopamine receptor antagonist, domperidone, prevented the prolactin-lowering effect of ergovaline. Therefore, the effects of ergot alkaloids in milk production and agalactia can probably be explained by their effect on dopamine D$_2$ receptors.

3. EFFECT OF ERGOT ALKALOIDS ON THE IMMUNE SYSTEM

Immunologists generally agree that recent evidence suggests a balance between prolactin (PRL) and glucocorticoids is important in regulating the immune response. Numerous studies have reported a reduction in PRL levels when ergot alkaloids are consumed (reviewed by Cross et al., 1995). Natural killer cell activity is suppressed by several dopamine receptor antagonists (huxthine, fluphenazine, pimoicde, and halopeudal, in a PRL-dependent manner (Fiserova and Pospisil, 1999). Fiserova and Pospisil (1999) theorized interaction between ergot alkaloids and lymphoid cells and tumor cells based on indirect findings: (1) expression of dopamine, serotonin or $\alpha$-adrenoreceptors or lymphocytes that can be involved in the action of ergopeptines; (2) inhibition of signaling pathways through intracellular enzymes (serin/heronin kinases, Ras-MAPK), leading to mitogenesis by activation of the adenylate cyclase system (D$_2$ agonistic, PRL inhibitory ergolines); (3) interaction with the nuclear structures or direct binding with DNA, including derivatives of agroclavine, festuclavine, and several ergopeptines—LSD, ergosine, ergosinine, and dihydroergosine.

4. TOXICITY OF THE ERGOT ALKALOIDS OF NEOTYPHODIUM COENOPHIALUM

4.1. Neotyphodium coenophialum Toxicosis in Cattle

Tall fescue grass contains an endophyte fungus (Neotyphodium coenophialum). Signs of fescue toxicosis in cattle that have been reported are increased body temperature, fescue foot, reduced weight gain, fat necrosis, diminished milk production and serum prolactin, and reduced reproductive performance (Schmidt and Osborn, 1993; Porter and Thompson, 1992; Thompson and Stuedemann,
Tall fescue toxicosis causes millions of dollars of loss in the animal industry and therefore has been studied extensively.

4.1.1. Body Temperature

_Blood Flow._ Increased body temperature has been exhibited in many species that ingest tall fescue pasture, hay, or seeds infected with _Neotyphodium coenophialum_ (Aldrich et al., 1993a; Williams et al., 1984; Spiers et al., 1995; Daniels et al., 1984). This increased temperature is thought to be a result of peripheral vasoconstrictive properties of the alkaloids found in tall fescue (Schmidt and Osborn, 1993; Porter and Thompson, 1992; Thompson and Stuedemann, 1993). The normal flow of blood seems to be shunted away from the peripheral tissues and restricted to the body core, thereby, depriving tissues in the extremities of much needed oxygen and nutrients. The restriction of blood flow to the peripheral tissue also takes away a very important cooling mechanism. Since blood flow to the peripheral tissues is drastically reduced, heat dissipation from evaporative cooling is much less effective. Rhodes and co-workers (1991) found that steers consuming a high-endophyte diet had reduced blood flow to the skin covering the ribs, the cerebellum of the brain, the duodenum, and the colon. Additionally, rectal temperatures were higher in this group of steers. Another study, conducted by Osborn and co-workers (1992), reported that Holstein steers consuming endophyte-infected (E+) fescue or (E−) fescue with ergotamine tartrate added at 30 ppm had decreased ear canal, pastern, coronary band, and tail tip temperatures, and increased rectal temperatures. These results agree with the hypothesis that physiological peripheral vasoconstriction results in increase shunting of blood to the body core.

4.1.2. Milk Production and Prolactin Levels

Consuming endophyte-infected tall fescue decreases serum prolactin and milk production in several species; however, the severity varies among species. Cattle, sheep, and rats show depressed milk production, while horses, llama, and rabbits show total agalactia (Cross et al., 1995).

Beef cattle at the Black Belt Experiment Station in Alabama (USA) showed a 43% reduction in milk yield, and weaned calves were 50 kg lighter than cows on endophyte-free pasture (Hill et al., 1985). Peters et al. (1992) reported a 25% reduction in milk yield of cows grazing endophyte-infected Kentucky 31 fescue when compared to noninfected Mossark fescue or orchard grass pastures. Brown et al. (1993, 1996) reported a decrease in milk production in cows grazing tall fescue as compared to common Bermuda grass. Numerous other experiments show reduced serum prolactin levels in steers (Aldrich et al., 1993a; Porter et al., 1990; Schillo et al., 1988; Thompson et al., 1986), heifers (Aldrich et al., 1993a; Porter et al., 1990), and cows (Mizinga et al., 1990; Wallner et al., 1983).
4.1.3. Reproductive Performance

Since most of the tall fescue is consumed on commercial cow–calf operations (Schmidt and Osborn, 1993), one of the most economically devastating aspects of fescue toxicosis in cattle is reduced reproductive performance. Lower conception rates mean a smaller calf crop. With negatively affected milk production and poor or slow calf weight gains, the problem is compounded. Schmidt and co-workers (1986) grazed heifers on low (0–5% of plants infected), medium (25–60% of plants infected), or high (80–99% of plants infected) levels of E$_+$ fescue. They were grazed over the winter and bred in the spring. Pregnancy rates decreased with increasing infection rate of the pasture and were 96%, 82%, and 55% for low, medium and high infection rates, respectively. During the second year that cattle were on these pastures, conception rates were further reduced in all three groups. Ninety-three percent of low-infection-pastured cows were pregnant, while pregnancy rates for the medium and high-infection-rate cows were 45% and 33%, respectively. These researchers reported a 50% drop in milk yield from cows in the high-infection-rate group and noticed that calf weight was beginning to reflect that decrease by 100 days of age. Irwin (1997) found that cows grazing E$_+$ pastures and treated with a D$_2$ receptor antagonist, domperidone, had pregnancy rates of 75%, while untreated cows had pregnancy rates of 51%.

4.1.4. Growth Rate

Figure 1 summarizes the effect of E$_+$ fescue on postweaning growth rate in steers as summarized from published research reports in several states in the United States. These data attest to the consistent reduction in gain of steers grazing E$_+$ fescue, regardless of the location. When the results of these studies were combined, there was a mean 44% reduction in average daily gain of steers on E$_+$ fescue.

Feedlot performance of steers that previously grazed E$_+$ fescue was usually higher than that of cattle that previously grazed E$_-$ fescue, which was probably a compensatory gain response (Fig. 2). However, E$_+$ cattle that were shipped to Southern United States feedlots during hot summer months did not exhibit this positive compensatory gain response. One of the negative effects of E$_+$ fescue on subsequent feedlot performance appears to be the effect of E$_+$ fescue on the immune system. According to feedlot managers, E$_+$ cattle tend to get sick more often during the first few weeks in the feedlot. However, the E$_+$ cattle that do not get sick perform better than cattle coming from non-E$_+$ pastures.

Most of the tall fescue pastures are grazed by beef cows. Figure 3 shows the effect of E$_+$ fescue on cow gain. Mean cow gain was 0.25 and 0.02 kg/cow/day for E$_+$ and E$_-$ cows, respectively.
**Figure 1** Postweaning average daily gain (kg) of steers consuming E+ and E− fescue. (Data of Hoveland et al., 1983; Schmidt et al., 1982; Goetsch et al., 1988; Studeman et al., 1986; Boling, 1985; Evans et al., 1989; Crawford et al., 1989; McMurphy et al., 1990; Chestnut et al., 1991; Read and Camp, 1986; Tulley et al., 1989.)

**Figure 2** Feedlot gain of steers that previously grazed fescue. (Data of Cole et al., 1987; Piper et al., 1987; Lusby et al., 1990; McDonald et al., 1988; Smith et al., 1986.)
4.2. *Neotyphodium coenophialum* Toxicosis in Horses

For many years, veterinarians and horse owners reported reproductive problems in mares that consumed tall fescue (Garrett et al., 1980; Villahoz et al., 1984; Poppenga et al., 1984). Monroe et al. (1988) determined that the endophyte of tall fescue is the causative agent for reproductive abnormalities in gravid mares (Fig. 4). Monroe reported that increased gestation lengths, agalactia, foal and mare mortality, tough and thickened placentas, weak and dysmature foals, and reduced serum prolactin and progestogen levels occurred in mares consuming endophyte-infected (E+) pasture, whereas horses on endophyte-free (E−) pasture appeared normal.

4.2.1. Gravid Mares

*Increased Length of Gestation.* Gestation length of mares increased 27 days when consuming E+ fescue grass, compared to mares consuming E− grass (Monroe et al., 1988). Putnam et al. (1991). Earle et al. (1990), and Redmond et al. (1994) observed similar results. Severe dystocia is a frequent observation in mares that try to foal after the extended gestation period. Supplementing E− and E+ mares on pastures with 50% of their NRC requirements for energy, using whole shelled corn, provided no beneficial effects on length of gestation or dystocia. In this study, 66% of the E+ mares with energy supplementation exhibited prolonged length of gestation and died due to dystocia, while 50% of the E+ mares without supplementation exhibited prolonged length of gestation and death due to dystocia. Putnam et al., (1991) reported that 10 of 11 mares on E+ fescue
experienced obvious clinical dystocia, and only one foal survived the natal period.

The dystocia appears to be a result of inadequate preparation of the reproductive tract for foaling, prolonged gestation, and fetal malpresentation. Due to prolonged gestation, foals usually have larger-than-normal skeletal frames, increasing the difficulty of expelling a fetus through an unprepared tract (Monroe et al., 1988; Putnam et al., 1991; Redmond et al., 1991a). Additionally, foals are often rotated 90–180° from the normal position for delivery (Taylor et al., 1985; Monroe et al., 1988; Redmond et al., 1991a). The failure of the mare, or the foal, to initiate the events that prepare for and result in normal parturition results in the subsequent catastrophic events of dystocia, as well as mare and foal mortality in some instances.

Agalactia. The effects of endophyte consumption on milk production varies among species. Cattle (Strahan et al., 1987; Porter and Thompson, 1992; Schmidt and Osborn, 1993), sheep (Stidham et al., 1982), and mice (Zavos et al., 1988) have been shown to have reduced milk yields, whereas horses (Monroe et al., 1988; Fig. 4) and rabbits (Daniels et al., 1984) exhibit reduced milk yields or complete agalactia. The connection between tall fescue toxicosis and

![Figure 4](image)

**Figure 4** Effect of E+ versus E− fescue on gestation length, foal mortality, agalactia, incidence of placental retention, and rebreeding response in mares. Stairs indicate difference between treatments ($P < 0.05$). (Adapted from Monroe et al., 1988.)
lactogenesis seems to be through the effects of the ergot alkaloids on lactogenic hormones. Cattle, sheep, and mice have both placental lactogen and prolactin (Forsyth, 1986). In contrast, horses and rabbits rely on prolactin to stimulate prepartum lactogenesis (Forsyth, 1996). The depressive effects of the ergot alkaloids on prolactin secretion may suppress prolactin’s effect on lactogenesis in cattle, sheep, and mice, but have little or no effect on placental lactogen. Consequently, the placental lactogen and the small level of pituitary prolactin may be sufficient to initiate prepartum lactogenesis in these species and allow lactation to begin after parturition. In the horse, it seems that the reduced prolactin secretion from the pituitary lactotrophic cells results in agalactia. The alkaloids of tall fescue are serving as D₂ dopamine receptor agonists at the pituitary level (Strickland et al., 1992). Also, unlike ruminants, the horse does not benefit from pregastric metabolism of alkaloids and would be subject to absorption of larger amounts of the alkaloids from E+ tall fescue (Wachenheim et al., 1992).

Eighty-eight percent of E+ mares were agalactic when maintained on fescue up to foaling (Monroe et al., 1988; Fig. 4). The milk of agalactic mares often appears as a brown or straw-colored oily-looking fluid, rather than the white milk of normal mares. This fluid has little nutritional value and foals invariably die unless bottle-fed. Another frequent complication affecting foal viability is the lack of normal immunoglobulins in foals from mares that produce the straw-colored fluid rather than white milk (Kouba et al., 1995).

**Placentas.** The placentas of mares grazing E+ tall fescue are thickened, reddish colored, and heavier, with an increased rate of retention than for E− mares (Monroe et al., 1988). Using an Ingstrom meter to measure stress and strain, these E+ placentas appeared to be more resistant to force that would tear them, which partially explains why some foals are unable to break through the thickened placentas (Monroe et al., 1988). Frequently, the foal is presented normally but encased in a tough, thickened chorioallantois membrane, which it cannot break through, and it therefore suffocates unless an attendant is present to cut the chorioallantois immediately.

Taylor et al. (1985) reported heavier and thicker placentas from mares consuming E+ seed than from mares consuming E− seed. DNA, RNA, and collagen content were greater in the placentas of mares consuming E+ seed. Caudle and Miller (1990) reported placental edema, placentitis, and mineralization of placentas from mares grazing E+ pastures. Brendemuehl et al. (1994) grazed mares on E+ fescue either continuously, from 300 days of gestation to foaling, from gestation day 60 to 300, or no exposure to fescue at all. They observed an increase in weight and width of the combined chorioallantois from mares exposed to E+ fescue continuously or from day 300 to foaling. Brendemuehl (1994) reported increased placental thickness in E+ mares immediately before parturition. Using 12 E+ and 12 E− mares, increased
placental thickness was observed in one E+ mare, 32 h prior to parturition, with 10 of the other mares demonstrating an increase in thickness with a mean of 6.5 h prior to the onset of parturition. An elective Cesarean section was performed in one E+ mare at 358 days of gestation and within 2 h of noting an increase in placental thickening. At surgery, the placenta was reported to be thickened in a plaquelike fashion in the ventral portion of the gravid horn. The thickened portion of the chorioallantois was noted to be separated from the uterus. Premature placental separations are common during the last trimester for mares grazing E+ fescue and are commonly referred to as “red bagging.” Frequently these mares develop their udders prematurely and can leak milk.

*Foal Vigor and Viability.* Monroe et al. (1988) observed large-framed, dysmature, and emaciated-looking (poor muscle mass) foals with overgrown hooves in E+ mares whose foals survived the birthing process and whose average gestation length was 27 days past the expected foaling date (Fig. 4). These foals appeared weak and many times exhibited “dummylike” behavior. Later, with proper care, the foals appeared normal (Monroe et al., 1988; Earle et al., 1990). Septicemia is a frequent problem and is likely a result of the low level of passive immunity. Putnam et al. (1991) reported that for 11 mares grazing E+ fescue, only three foals were alive at birth, and only one of the three survived the first month of life. Dysmaturity or neonatal death of foals was not observed in 11 mares grazing E− pastures.

Taylor et al. (1985) and Kosanke et al. (1989) observed lack of lung maturation in stillborn foals born to E+ mares. Amniotic fluid from E+ mares lacked pulmonary phospholipids, and phosphatidylethanolamine was present in only 12% of E+ mares (Clare et al., 1994). These data suggest that lack of lung maturation may be a contributing factor to the high rate of foal death observed in E+ mares. Boosinger et al. (1994) examined several organs and tissue from foals of E+ and E− mares. Histological studies of thyroid glands from foals exposed to E+ continuously or after gestation day 300 revealed numerous distended colloid-filled thyroid follicles lined by flattened cuboidal epithelial cells. Mean plasma T3 concentrations were reduced in these foals. Foals from mares exposed to E+ continuously or from day 300 to foaling demonstrated a response to thyroid-stimulating hormone (TSH) by showing improved mental alertness, desire to stand, and good suckle reflex.

Brendemuehl (1995) collected colostrum from normal mares and tested it for IgG concentration. Foals from mares exposed to E+ fescue continuously or from gestation day 300 were administered 11 of the pooled colostrum collections by nasogastric tube within 1 h of birth. Compared to control foals, these foals had decreased serum IgG concentrations. These data combined with other data suggest that foals from E+ mares receive less IgGs from the mare’s milk and absorption rate is lower even if the milk IgG levels are at or near normal levels. These factors, combined with the lower level of colostrum production in E+
mares, explain why many foals from E+ mares quickly become septic. This, along with the low nutrient intake from milk, probably accounts for many foal deaths in E+ mares with live foals at birth. Brendemuehl et al. (1994) observed lower serum T3, ACTH, cortisol, and total progesterone levels in foals from E+ mares compared to foals from E− mares.

Body Temperature, Blood Flow, and Laminitis. In cattle and sheep, blood flow to the peripheral tissues decreased and body temperature increased when tall fescue seed was included in the diet (Rhodes et al., 1991). The reduction in blood flow to the peripheral tissues is likely related to increased body temperature, because the animal is less efficient in cooling itself. Unlike cattle and sheep, pregnant mares exhibit no increase in body temperature when exposed to the endophytic toxins (Monroe et al., 1988; Putnam et al., 1991). However, horses sweat more freely than cattle and are more capable of cooling themselves. Putnam et al. (1991) observed increased sweating in gravid mares grazing E+ tall fescue.

In cattle, it seems that peripheral vasoconstriction caused by the alkaloids of E+ tall fescue is related to “fescue foot” (Solomons et al., 1989). Rorhback et al. (1995) reviewed data from 185,781 horses, of which 5536 had a diagnosis of laminitis. Although these data are preliminary, they concluded that there appeared to be a relationship between laminitis in horses and consumption of E+ fescue grass. Abney et al. (1993) observed a vasoconstrictive effect of ergot alkaloids on equine vessels in vitro. Carbohydrate overload (Gerner et al., 1975) and aqueous extract of black walnut (Galey et al., 1990) are associated with the development of laminitis in horses. The aqueous extract of black walnut caused postcapillary venoconstriction, increased capillary hydrostatic pressure, and transvascular fluid movement, resulting in increased tissue pressure, edema, vascular collapse, and ischemia in the equine digit (Eaton et al., 1995). It is possible that through interaction of the ergot alkaloids with the adrenergic receptors of the sympathetic nervous system, similar responses may be occurring in horses consuming E+ fescue. Direct evidence for this theory does not exist.

Mare Abortions and Fertility. Abortions in mares occur after rapid separations of the placenta from the endometrium. Of 1211 abortion/stillbirths presented to a diagnostic laboratory in Kentucky (USA), placentitis and dystocia were the commonly diagnosed causes (approximately 11%, each), with congenital abnormalities (8%), twins (6%), umbilical cord torsion and premature placental edema (4%, each), and equine herpes virus and other bacterial infections (3%, each) being the other diagnostic causes (Pugh and Chapman, 1996). “Red bagging,” or premature placental separations, and stillborn foals are frequently reported by veterinarians in the field for mares grazing E+ fescue. Good quantitative data on the abortion rate of mares past 30 days of gestation does not exist.

Brendemuehl et al. (1994) observed the effects of E+ fescue on mare cyclicity, pregnancy rates, and embryonic death rates. Mares grazing E+ pastures
demonstrated prolonged luteal functions, decreased per-cycle pregnancy rates, and increased early embryonic death rates compared to those grazing E− pastures.

4.2.2. Growing Horses

**Effects on Growth Rate and Digestibility.** Consumption of E+ tall fescue or treatment with its extract causes a reduction in rate of gain and feed intake in cattle (Schmidt et al., 1982; Hoveland et al., 1983; Bond and Bolt, 1986), rats (Neal and Schmidt, 1985), and rabbits (Daniels et al., 1984). No reduction in growth rate was observed in yearling horses when corn-based concentrates were used to supplement E+ or E− hay (McCann et al., 1992). Also, Pendergraft and Arns (1993) observed similar gains in yearling horses consuming E+ or E− hay with concentrate supplementation to meet NRC requirements for growth. However, average daily gains were reduced by 57% (0.24 and 0.56 kg for high- and low-endophyte treatments, respectively) in yearling horses grazing E+ pasture without supplementation, with a similar reduction in gain for steers in the same study (Aiken et al., 1993).

Redmond et al. (1991b) and McCann et al. (1992) observed lower intake and digestibility for E+ hay fed to mature geldings and yearling horses, respectively. McCann et al. (1993) and Pendergraft and Arns (1993) found no differences in digestibility due to the presence of the endophyte in hay when yearling horses were fed concentrate with hay. Concentrate supplementation was used in both studies to meet NRC requirements for growth for yearling horses.

These results suggest that the effects of endophyte consumption on digestibility and growth rate may be lessened by the inclusion of concentrates in the diet. In contrast, energy supplementation has no beneficial effects for alleviating lactation and reproductive problems in gravid mares that graze E+ pasture (Earle et al., 1990).

5. MANAGEMENT AND TREATMENT OF *NEOTYPHODIUM COENOPHIALUM* TOXICOSIS

5.1. Pasture Management

Personal interviews of horse owners and veterinarians and clinical research has revealed that many horses exhibit many of the symptoms of E+ toxicosis while consuming only small quantities in hay, small patches of E+ fescue hay in paddocks, or even by grazing a small quantity of E+ fescue under paddock fences (Cross et al., 1999). Therefore, pastures must be completely rid of E+ fescue to prevent toxicosis in horses. Personal experience and interviews with livestock owners throughout the United States attest to the extreme difficulty of ridding pastures of E+ fescue. Experience has shown that unless pastures are completely
devoid of E+ plants and viable seed, the E+ plants begin to thrive and become significant problems within 1 to 3 years after replanting of pastures. Best success with pasture reseeding has come through the use of chemical killing of the fescue sword, followed by aggressive choke crops for 2 years before reseeding is attempted. Establishment of clover or other forage mixes with E+ fescue seems to be a reasonable alternative for cattle, but not for horses.

5.2. Grazing Behavior

The horse is a notorious selective grazer and will select many alternative forage species before consuming E+ fescue. Under low grazing pressures, many mares will spot-graze other species of forage and never exhibit any signs of fescue toxicosis. Changes in grazing pressure or availability of alternative forages can quickly force E+ fescue consumption and the classical signs of fescue toxicosis. This partially explains why some horse owners appear to have little or no fescue toxicosis when a few mares are grazing a large acreage of mostly E+ fescue, and other horse owners routinely have problems.

5.3. Removal of Mares from E+ Pastures

There is evidence to suggest that the effects of E+ fescue are greatly reduced if mares are withdrawn from E+ pastures at least 30 days prior to expected foaling (Taylor, 1993). Brendemuehl (1995) arrived at similar conclusions. However, most veterinarians recommend removal of mares from E+ pastures from 30 and up to 90 days prior to expected foaling; with this approach, “red bagging” and the other signs of fescue toxicosis are greatly minimized.

5.4. Therapeutic Treatment

5.4.1. Dilution of Toxin Intake

Gravid mares were fed 50% of the NRC requirement for energy as cracked corn for the last 90 days of gestation (Earle et al., 1990). There were no beneficial effects as a result of grain feeding. Foal mortality was 66% and 100% for the energy and no energy supplement treatments, respectively. This study confirmed the severity of the problems under the conditions in the Southeastern United States.

5.4.2. Dopamine Antagonists

Strickland et al. (1991, 1994) studied the effects of ergot and loline alkaloids of E+ fescue on prolactin release by isolated and perfused rat pituitary cells. The ergot alkaloids had prolactin-lowering effects and mimicked dopamine action. The use of a D2 dopamine receptor antagonist (domperidone) blocked the effect of the ergot alkaloids and prevented their prolactin-lowering effect.
is a D₂ dopamine receptor antagonist that does not cross the blood–brain barrier or elicit neuroleptic side effects. Domperidone was administered orally (1.1 mg/kg body weight) to gravid mares grazing E+ tall fescue (Redmond et al., 1994). Domperidone increased serum prolactin and progestogens and provided what seemed to be nearly complete recovery of gravid mares from tall fescue toxicosis, without neuroleptic side effects. Treated mares had milk, live, healthy foals, and gestation length similar to the calculated gestation length. Subsequently, a dose titration study (Figs. 5–7) was conducted to determine the minimum effective dose of domperidone for treating tall fescue toxicosis (Redmond et al., 1993). Again, domperidone provided recovery from tall fescue toxicosis in gravid mares, and the minimum effective oral dose was 1.1 mg/kg body weight when administered daily for 30 days before foaling.

In a field study (Cross et al., 1999), domperidone was administered to 1423 periparturient mares in several states in the United States. Veterinarians and/or

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**Figure 5** Effects of varying levels of domperidone (1.1, 1.65, or 2.2 mg/kg, PO SID) on mammary gland development in periparturient mares grazing E+ fescue pastures. Day – 31 represents the average number of days prior to the calculated date of parturition that pretreatment samples were obtained (pretreatment samples obtained 1.2 days before initiation of drug treatment). Stars indicate first detectable difference (P < 0.05) from pretreatment values within treatment. Numbers indicate number of animals remaining in treatment group when sample was obtained. (From Redmond, 1994.)
horse owners reported the drug to be 94.5% effective in prevention of the signs of fescue toxicosis. Mares consuming the drug in a preventative mode did not experience increased gestation, dystocia, agalactia or lower-than-normal milk production, retained placentas, premature placental separations, or the dead, weak, or dysmature foals that had been observed in nontreated control mares.

5.4.3. Mechanism of Action of Domperidone for Treating Equine Fescue Toxicosis

Domperidone’s action as a D₂ dopamine receptor antagonist prevents the ergot alkaloids from mimicking dopamine’s actions. The most apparent action of dopamine and the ergot alkaloids of fescue is their prolactin-lowering effect. With administration of domperidone to E+ mares, prolactin is returned to normal levels and even increased above normal levels in most instances (Redmond et al., 1993). Certainly prolactin is a major player in equine fescue toxicosis, but as is
evidenced by the preceding review of endocrine effects of E+ fescue, prolactin is one of many hormones altered. Prolactin, along with the progestogens and estrogen, are certainly major players in the milk production maladies observed in E+ mares, and administration of domperidone returns these hormone levels to near-normal levels. Also, domperidone’s effect on peripheral circulation as an \( \alpha-1 \) receptor antagonist provides evidence for its remediation of the negative microcirculatory effects of the ergot alkaloids in E+ fescue.

Also, since the hypothalamic–pituitary–adrenal axis (HPA) of the fetus in E+ mares appears to be compromised and results in prolonged gestation lengths in mares, and the associated problems thereof, domperidone may be having some effect on the HPA system since mares receiving domperidone while grazing E+ fescue foal at or near their expected foaling date with normal, healthy foals. And, since ACTH levels in foals from E+ mares are low, and ACTH is the stimulus for adrenal cortisol release and since normal fetal adrenal cortisol levels appear to be

**Figure 7** Effect of endophyte-infected fescue and domperidone (1.1, 1.65, or 2.2 mg/kg, PO, SID) treatment on serum progestogen levels in gravid mares. First detectable differences \((P < 0.05)\) from pretreatment levels are indicated by stars. Unless otherwise indicated, data points represent four mares per treatment (dagger indicates number of mares in E group). Mares which were not showing signs of impending parturition 7 days after the calculated date of parturition (as determined by veterinary examination) were relocated to endophyte-free pasture. (From Redmond, 1994.)

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necessary to trigger parturition, it is interesting to speculate that domperidone may be affecting this system. Zerbe et al. (1993) administered domperidone to dogs and observed an enhanced ACTH response to CRH injections. Thus, domperidone could be reversing the effects of E+ fescue on gestation length by effecting an increase in adrenal cortisol through CRH-stimulated release of ACTH. Direct evidence to confirm this theory does not exist.

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Commercial Applications of Endophytic Fungi

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1. INTRODUCTION

“Choke” is a condition in grasses caused by the endophytic fungus *Epichloe typhina*. The name choke is used because the characteristic mycelium starts within the host plant (endophytically), but emerges to form a visible reproductive structure around the seedhead that prevents flower development and seed production by the host. Visually, this mycelium structure appears to strangle or choke the host seedhead. Some grass species, however, serve as hosts to a related endophytic fungal genus called *Neotyphodium*. *Neotyphodium* species, whose mycelia never emerge, have no reproductive stage but rather are propagated by mycelia through the seed of the plant host and are not normally detrimental to the host. *Neotyphodium* species are highly evolved and classified as mutualistic rather than pathogenic.

Recently, the agricultural importance of these endophytes, especially *Neotyphodium*, has been recognized. For example, tall fescue (*Festuca arundinacea* Schreb.) is grown on more land area (by some estimates, over 20 million hectares) than any other perennial temperate forage grass species in
the United States (Bouton, 2000), far outweighing other pasture grasses in importance and use. The vast majority of this tall fescue acreage is infected with Neotyphodium coenophialum. This amount of acreage is more than enough to support the hypothesis that $N. \text{coenophialum}$ is critical for the persistence and increased “ecological fitness” of this grass species (Bouton, 2000). However, this infection leads to “fescue toxicosis,” a toxicity that is generally characterized by unthrifty appearance as well as poor levels of weight gain and reproduction (Stuedemann and Thompson, 1993), in ruminant animals grazing the forage, as a result of ingestion of ergot alkaloids derived from the endophyte association (Hill et al., 1994).

The toxicity of endophyte-infected ($E^+$) tall fescue, therefore, presents livestock producers with a dilemma of whether to grow $E^+$ cultivars for stand persistence, but risk reduced animal performance due to the inherent toxins. Since the vast majority of tall fescue acreage in the United States, including newly planted areas, is $E^+$, most producers have decided that stand persistence, and not fescue toxicity, is the most important consideration in their operation. In a survey by Lacefield et al. (1994), 80% of current tall fescue acreage was found to be infected with $N. \text{coenophialum}$ at a mean infection rate of 76%, and 50% of the respondents considered their new plantings with endophyte-free ($E^-$) cultivars to be failures. In the same survey, it was found that the main reasons farmers do not convert to $E^-$ pastures were lack of confidence in $E^-$ cultivars and a perception that the benefits do not outweigh costs. The failure of $E^-$ cultivars to assume a substantial share of the tall fescue seed market also supports the view of the on-farm importance of stand persistence. It goes without saying, therefore, that most livestock grazing tall fescue pastures in the United States probably suffer from some degree of fescue toxicity.

A very similar situation exists for the widely planted pasture crop, perennial ryegrass, Lolium perenne L., in New Zealand and Australia (Fletcher and Easton, 2000; Reed et al., 2000), and probably Europe (Lewis, 2000). Perennial ryegrass is naturally infected with $N. \text{lolii}$, giving the plant several agronomic advantages, but the presence of ergot and tremorgenic alkaloids can cause severe problems in both cattle and sheep.

The positive agronomic characteristics of these endophytic associations have recently led to efforts to commercialize them. The ability to find natural associations, coupled with an ability to identify and maintain them in plant germplasm collections (Clement, 2000), will continue to be important. Current commercialization efforts have pursued several strategies and will potentially increase in both scope and number in the future.
2. STRATEGIES

Before embarking on a program to commercialize fungal endophytes, the initial questions are whether the grass species possesses a natural association and, if so, whether this association is beneficial to the grass host. As mentioned above, the main grass species/fungal endophyte associations with commercial value are currently the tall fescue/N. coenophialum and the perennial ryegrass/N. lolii associations. This is because the positive effects of endophyte infection in both turf and pasture situations are well documented and accepted for these two associations. However, there are many documented associations of different grass species with Epichloe, Neotyphodium (synonym = Acremonium) endophytes (Table 1). The ecological benefit of these natural endophyte infections on many of these species is still not clear or not evolved to the level of tall fescue and perennial ryegrass. In those important grass species where there is no or limited benefit for the host, questions to ask before commercialization are whether endophytes with current value (e.g., N. lolii or N. coenophialum) possess cross-compatibility with these grasses or whether they can be engineered to do so. Another question is whether their endemic endophyte species can be engineered to give benefits when reinfected into the host.

Current strategies involve enhancing the positive benefits to the plant and developing the best host compatibility possible. There are two main ways to accomplish this. First, the endemic endophyte(s) can be left in the host plant and the plant and endophyte combination selected for value-added traits naturally within the plant germplasm, and/or the traits can be incorporated directly into the association via plant hybridization or genetic engineering (e.g., plant breeding). Second, the endophyte can be removed from one host and reinfected into the same or another host. This process requires that the endophyte contain the least amount of undesirable characteristics, or changing undesirable traits in the endophyte through genetic engineering or mutagenesis. The plant germplasm/endophyte associations resulting from either of these two strategies must then be extensively reselected and tested for their commercial potential.

2.1. Leaving Endophyte in Host Plant

Assessing the characteristics of endemic associations and using host plant phenotypic selection to enhance desirable traits and reduce undesirable traits would require the least amount of resources, technology, and manipulation. In actual practice, there is simultaneous selection pressure on both the plant and endophyte strain(s) and for all genes needed for a successful association.

2.1.1. Toxic Associations

The most common approach has been either to consciously (or unconsciously) leave a toxic endophyte in the germplasm after determining that infection is
<table>
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<tr>
<th>Host</th>
<th>Endophyte</th>
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<th>Negative effects</th>
<th>Neutral effects</th>
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<td><em>N. coenophialium</em>(^b)</td>
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<td><em>A. spp</em></td>
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\(^a,b\) Species have been identified using the most recent taxonomic information. Where applicable, species names have been updated from those used by the original authors.
beneficial, or to conduct normal plant breeding selection procedures for traits of agronomic importance within toxic E+ associations. The best and most widely used examples have been the management, with techniques designed to minimize toxicity, of the many acres of E+ Kentucky 31 already in existence (Ball, 1997) and the recent development of E+ tall fescue and perennial ryegrass turf cultivars with better persistence, where animal toxicities are not a problem (Funk and White, 1997). The tall fescue turf cultivars were generally grouped and sold as “endophyte enhanced.”

For forage cultivars, the recent trend has been to develop only E− cultivars, but this approach has been limited from a commercial point of view (Bouton, 2000). However, in the lower southern United States, where tall fescue is not currently used due to poor stand survival, E+ germplasms selected for persistence have better plant survival (Bouton et al., 1993a). Since the cost of wintering cattle in this region with hay is costly and poor from a nutritional standpoint, it was documented that even using E+ tall fescue, when grown in mixtures with bermuda grass and bahia grass, gave good winter gains on beef heifers with no hay feeding; animal toxicities were acceptable under these management conditions (Gates et al., 1999). These findings resulted in the release of “Georgia-5” tall fescue as an E+ cultivar for use in this region of the country (Bouton et al., 1993b).

Another successful example of leaving the endemic toxic endophyte in the host plant is selection for reduced alkaloid production within toxic tall fescue/\textit{N. coenophialum} associations. This approach was found to be highly heritable (Adcock et al., 1997). One germplasm selected for low ergot alkaloid production from the E+ version of the “Jesup” cultivar (Bouton et al., 1997), Jesup “961,” was tested in animal and agronomic performance trials and found to show ergot alkaloid concentrations nearly 70% less than Jesup (E+) (N. S. Hill, personal communication, 1998). The Jesup (E+) check and Jesup “961” were also shown to have superior stand survival when compared to Jesup (E−), but Jesup “961” did show a reduced stand survival when compared directly to Jesup E+. Animals grazing pasture with the low-alkaloid germplasm demonstrated weight gain between that of animals on E+ and E− pasture, but possessed serum prolactin levels more like animals on E+ forage (Bouton, 2000). These studies demonstrated that persistence and alkaloid concentration were stable over environments, but the lingering symptoms of animal toxicity as seen with depressed blood prolactin and the stand reduction exhibited by the low-alkaloid-producing germplasm raise questions about this strategy of breeding for reduced rather than nil toxic alkaloids.

2.1.2. Nontoxic Associations

Although no commercial product has resulted thus far, it is obvious that finding natural nontoxic associations within a grass species and using selection to
enhance the agronomic characteristics of the association, which is actually just a form of maternal line breeding, should be achievable (Reinholz and Paul, 2000). However, the approach of removing nontoxic endophytes for reinfection into already commercially viable plant cultivars was the initial strategy of choice (see below). This is because the vast majority of cultivars were already infected with toxic endophytes and the feeling was that by directly substituting nontoxic endophytes one would preserve the value of these already successful cultivars. More important, it also allowed the endophytes to be viewed as a patentable technology, thus greatly enhancing their profit potential. Since the first commercial products in tall fescue and perennial ryegrass are patented endophytes, any approach of finding natural, nontoxic associations and using plant selection and maternal line breeding to get a commercially viable endophyte/plant cultivar product will have the problem of proving that the endophyte in the final product is not already under patent protection. This will increase the cost of this approach in terms of time and money.

3. REMOVAL OF ENDOPHYTE FROM HOST PLANT FOR LATER REINFECTION

Removal of endophytes from the host plant for later reinfection has been the most widely used commercial approach because of the ability to capitalize on already successful cultivars and to handle the endophyte as a patentable technology. Patenting allows for the most successful commercialization because profit margins are potentially greatest.

3.1. Nongenetic Manipulations of Natural Endophyte

Reinfection of widely used or elite cultivars with naturally occurring, nontoxic endophyte strains, initially investigated in New Zealand for the perennial ryegrass/N. lolii association (Tapper and Latch, 1999), represents the most successful commercial approach to date. The recent work in the United States by Bouton et al. (2000) has also resulted in a commercial product for tall fescue. The key to this approach has been the successful employment of the reinfection techniques first investigated by Latch and Christensen (1985) and the ability to obtain patent protection for the endophytes themselves.

3.1.1. Perennial Ryegrass

In the New Zealand work with perennial ryegrass, endophyte strains incapable of producing lolitrem B, the toxic alkaloid responsible for “ryegrass staggers” in sheep, but still capable of producing peramine for deterrence of the Argentine stem weevil, the main insect pest of perennial ryegrass in the country, were isolated and reinfected into two cultivars (Tapper and Latch, 1999). The most
successful strain, sold as “Endosafe,” was resistant to Argentine stem weevil, and did not cause ryegrass staggers. However, Endosafe was found in one of the cultivars, Grasslands Pacific, to produce ergovaline, which is felt to be the main ergot alkaloid responsible for fescue toxicity (Lane, 1999). The combination producing ergovaline also gave rise to some symptoms of fescue toxicity in cattle and was subsequently removed from the market. However, the other cultivar, Grasslands Greenstone Endosafe, did not produce toxic levels of ergovaline and is still sold in New Zealand. The more recent strains, in addition to nil production of lolitrem B, are also nil producers of ergovaline. One of these new strains, AR1, infected into several perennial ryegrass cultivars, is currently undergoing testing for potential commercialization, with positive results recorded thus far (Fletcher and Easton, 2000).

3.1.2. Tall Fescue

The main tall fescue cultivar used throughout the United States is still Kentucky 31, which was released in 1943 (Buckner, 1985). When infected with *N. coenophialum*, the growth, survival, drought tolerance, and competitiveness of Kentucky 31 is enhanced (Bouton et al., 1993a; Hill et al., 1991; West et al., 1993). The immense practical impact of these characteristics can be seen in a survey by Shelby (1991), in which pastures presumed to be E− demonstrated increasing levels of endophyte infestation of 4% per annum for 9–12 years. The increased infection percentage was attributed to better survival of infected plants in mixed stands during drought. However, the ergot alkaloids produced in infected plants nearly always result in poor weight gain and reproduction in animals consuming the forage. These effects on animal performance, first documented in Kentucky 31 by Hoveland et al. (1983), have been repeated for the same cultivar by other researchers (Stuedemann and Thompson, 1993) and recently for the cultivar Jesup (Hoveland et al., 1997). Based on the importance of endophyte infection in tall fescue, and the background information of using nontoxic endophytes described for perennial ryegrass, it was speculated that this same approach could be attempted for the tall fescue/*N. coenophialum* association (Latch, 1997; West et al., 1998).

In the first proof of concept studies, reinfection of two tall fescue cultivars, Jesup and Georgia 5, with a patented, but naturally occurring, nontoxic endophyte strain, AR542, was found to eliminate production of known toxic alkaloids and provide better lamb performance and pasture stand survival (Bouton et al., 2000). During the experiments backing up this effort, endophyte strains with very low production of total ergot alkaloids, yet capable of producing pest-deterring peramine and loline alkaloids, were isolated from field-grown plants collected throughout the world (Latch, 1993; G. C. M. Latch, 1994, personal communication). The objective of the work then became assessment of the strategy of reinfecting Jesup and Georgia 5 tall fescue with these nontoxic endophyte strains.
Different cultivar/nontoxic strain combinations were tested against the E+ and endophyte-free (E−) versions of the same cultivars for stand survival and dry matter yield at two locations, and in separate experiments, were assessed for toxicity in lambs (Bouton et al., 2000). The best combination, Jesup (AR542), produced no toxic ergot alkaloids and possessed stand survival better than the E− checks and equal to the E+ check. Georgia 5 (AR542) showed better survival than the E− check, but less survival and yield than the E+ check. For three spring (1998-2000) and two fall (1998-1999) grazing seasons, gains for lambs on both cultivars containing AR542 were equivalent to those from E− forage, but approximately 57% greater than animals on E+ forage. Animals consuming E− forage or forage from the nontoxic strain did not exhibit the depressed serum prolactin or elevated body temperatures of animals consuming E+ forage. Further testing with beef cattle, the primary commercial livestock group in the United States, also showed gains with the nontoxic strain, AR542, to be equivalent to gains on E− pastures, and much higher than gains in animals on E+ forage (Bouton, 2000). The AR542 strain was therefore successful in making a nontoxic substitution for the endemic toxic strain of both cultivars and will be marketed under the name of “MaxQ” (Bouton et al., 2000).

Overall, these studies demonstrated that reinfection of naturally occurring, nontoxic endophyte strains into elite tall fescue cultivars after removal of their toxic, endemic strain(s) is a good strategy for eliminating fescue toxicity and providing better animal performance yet keeping the agronomic characteristics desired by producers. A recent report of successfully isolating nontoxic strains and reinfecting them into other tall fescue germplasm also provides independent verification of the success of this strategy (West et al., 2001).

3.2. Genetic Manipulation of Endophytes

Although there are currently no commercial products, the use of “biotechnology” to improve endophyte strains and/or the endophyte/plant association has recently become an area of interest (Bacon and White, 1994). With the recent negative experiences of genetically modified organisms (GMOs) in important food crops, any work in this area must continue to monitor problems such as public perception, government regulation, and intellectual property issues. However, the biotechnologies are too powerful and useful not to be part of any future commercialization efforts.

The potential strategy of removing the toxic strains from successful tall fescue cultivars, using genetic engineering techniques to eliminate their toxic ergot alkaloid pathways, and reinserting them into viable cultivars was reported previously (Wilkinson and Schardl, 1997). Recent work on cloning genes responsible for the endophyte toxic pathways should allow for more efficient screening of natural nontoxic strains or development of strategies for eliminating
these genes in toxic associations (Scott, 2000). The ability to improve host plant cross-compatibility would also be an important area of investigation.

It has been recognized that fungal endophytes, because of their unique properties of living nonpathogenically throughout the grass host, can themselves be a vehicle for change. As such, they are viewed as vectors that are more easily manipulated than their genetically complex hosts. In this regard, Turner et al. (1993) proposed engineering the endophyte instead of the plant with genes that produce insect Bt toxins. With the growth of the mycelium throughout the plant, the plant would have insect deterrence capability without having these same genes inserted directly into its own genome.

4. PROBLEMS AFFECTING SUCCESSFUL COMMERCIALIZATION

4.1. Screening Techniques

Screening techniques to assess both presence and type (e.g., toxic or nontoxic) of endophyte are critical for successful commercialization. Such techniques must be accurate, rapid, highly repeatable, and capable of handling large numbers of plants. The initial techniques used, involving staining and microscopic methods to detect the presence of the endophyte and chromatography to assay the concentration of toxic alkaloids, were slow and expensive and have been replaced by more rapid and efficient immunological assays (Hahn et al., 2000). For example, the development of immunoblot procedures for detecting endophyte presence (Hiatt et al., 1997) and ELISA methods for quickly assessing presence of ergot alkaloids (Adcock et al., 1997) were critical for the background research in the commercialization of MaxQ. What evolved for the MaxQ program was a procedure (N. S. Hill, personal communication, 1997) whereby adjacent cross-sectional pieces of the lower pseudo-stem of an individual tiller were removed, with one piece being placed on the membrane filter for the immunoblot procedure (Hiatt et al., 1997) and the other crushed into the well of an ELISA plate which was then subjected to monoclonal antibodies specific for ergot alkaloids. Therefore, depending on the outcome of both assays, the plant could be scored as E−, E+ but possessing toxic alkaloids, or E+ but nontoxic. This greatly improved quality control issues during seed increase and allowed more efficient selection of the best reinfected genotypes and completion of the many agronomic and ecologically based studies to properly assess the abilities of MaxQ (Bouton et al., 2000). Use of detection techniques based on molecular markers such as restriction fragment length polymorphisms (RFLP) or microsatellites, because of their ability for specificity of detection, are likewise a potential area of growth.
4.2. Endophyte Viability

Maintaining endophyte viability during the breeding, seed production, marketing, and actual farmer establishment phases of commercialization will be the main challenge associated with strategies involving the introduction of specific endophytes into a given host. Endophytes appear to be very stable in planta, but fragile while in the seed. In most standard storage conditions, the endophyte has been found to die quicker than the seed embryo. Rolston et al., (1993) reported that low seed moisture (10% seed moisture content) and low temperatures (5°C) were effective in maintaining endophyte viability during seed storage.

Paul et al. (2000) reviewed the perennial ryegrass breeding process in most European programs and found three main causes for loss of endophyte infection in commercial cultivars. First, there is a dramatic loss of endophyte colonization of 5.5% per generation cycle during the generative or seed-increase phase. Second, phenotypic selection for “general appearance” desired by most European breeders resulted in loss of colonization density of 13% per cycle of selection. Third, the common practice of seed storage in open bins, without air conditioning, led to significant loss in endophyte infection and viability.

During development of MaxQ, the main initial selection criteria when infecting the Georgia 5 and Jesup cultivars, which are populations of different plant genotypes, were high infection and transmission rates of the reinfected strains during the seed-increase phase (J. H. Bouton, unpublished data, 1997). Initial seed production from 200 inoculated genotypes of each cultivar/strain combination was conducted in isolation glasshouses. This seed was then planted into a field isolation block. Tillers from these blocks showed differences among various cultivar/strain combinations for infection rate. For example, the AR572 and AR577 strains did not hold a high infection frequency in either cultivar, AR502 and AR510 maintained a high frequency in Jesup but not in Georgia 5, and AR542 transmitted and maintained equally well in both cultivars. It is assumed that any reduction of infection was not due to lack of transmission to offspring in either the initial increase in the glasshouse or the subsequent increase in the field isolation blocks, but rather due to an inability to maintain infection during seed conditioning and handling. All combinations, however, produced nil or near-nil levels of total ergot alkaloids, and maintained a high level of infection during all agronomic and animal toxicity trials. These results support the speculation that any reinfection of an isolated strain into a new cultivar will need to be intensely screened for compatibility and infection transmission and maintenance (West et al., 1998).
4.3. Testing

Since the main basis for any endophyte commercialization will be through a plant cultivar, the overall effort should be viewed as cultivar improvement. In any cultivar development effort, the testing phase is the most time- and resource-consuming process. For commercial forage cultivars with nontoxic endophytes, there is an added problem of needing both standard agronomic testing combined with trials of animal performance and/or toxicity.

4.3.1. Animal Trials

Typically, animal responses to the effects of tall fescue endophytic toxins can be grouped into four categories (Stuedemann and Thompson, 1993): (1) decreased weight gain and pregnancy rate, (2) behavioral criteria demonstrated with decreased feed but increased water intake, (3) physiological responses such as increased respiration and elevated rectal and core body temperatures, and (4) sera or plasma levels of constituents such as decreased serum prolactin and cholesterol. One or more of these responses are usually measured when assessing fescue toxicity (Stuedemann and Thompson, 1993). Similar types of studies were needed for perennial ryegrass infected with nontoxic *N. lolii* strains (Fletcher and Easton, 2000). Toxicity trials are therefore expensive and resource-consuming, but must be conducted if the commercial product will be sold as nontoxic.

4.3.2. Agronomic Trials

The main dilemma for livestock producers using tall fescue and perennial ryegrass is whether to grow current E+ cultivars for stand persistence but risk reduced animal performance due to the inherent toxins (Bouton, 2000; Fletcher and Easton, 2000). Since E− cultivars eliminate toxicity and are an alternative for producers willing to risk stand loss, any cultivar reinfected with an isolated, nontoxic endophyte strain must, at a minimum, provide better agronomic performance than E− cultivars in order to be a viable option for producers. The most successful combinations should provide the agronomic advantage of E+ cultivars. Agronomic testing therefore must be rigorous, examine the main environmental stresses for the region of interest, contain the best checks (e.g., E+ and E− entries), and be realistically applied and proven. However, trials may not be able to wait the many years required for completion of some studies. In this regard, Bouton (2001) documented that planting different tall fescue cultivars into bermudagrass (*Cynodon dactylon* L.), followed by livestock grazing, provided a realistic trial for quickly (less than 2 years) separating E+ and E− entries.

For example, in the evaluation of forage yield and stand survival during the MaxQ effort, the substitution effect of a nontoxic strain was more complex and variable than for the animal responses (Bouton et al., 2000). Of the three nontoxic
strains intensively tested in Jesup, only AR542 gave a nearly identical response to the E+ version for dry-matter yield and stand survival. Georgia 5 (AR542), while performing better than Georgia 5 (E−) for stand survival, produced less dry-matter yield in some instances, and had less stand survival during the second year, when compared to Georgia 5 E+. These responses are interpreted as meaning any new cultivar reinfected with even a previously successful strain such as AR542 will need to be intensely tested for agronomic performance before release into the commercial seed trade.

Besides measuring broadly based environmental variables, there may be a need to conduct trials that assess response to specific stresses, such as insects, diseases, and nematodes, that can individually lead to poor plant survival and performance. For example, although the AR1 endophyte when placed in commercial perennial ryegrass cultivars has met the general criteria for success such as acceptable animal and agronomic performance, it has shown some susceptibility to African black beetle (Fletcher and Easton, 2000). This has caused any commercialization effort to be combined with an educational effort so that farmers know they may have to control African black beetle in areas where this pest is a problem.

4.4. Intellectual Property Issues and Cost

Endophytes are viewed as a technology, actually the first commercial technology to be placed in grass cultivars, and as such can be patented. Currently, there are several endophytes under patent protection in New Zealand, Australia, and the United States, with Endosafe and MaxQ being paramount among them. As a protected technology, endophytes are expensive to bring to the marketplace and should be viewed in much the same way as the current herbicide and insect-resistance genes now being marketed in many row-crop cultivars. However, the grass seed industry is currently a commodity-type business, with most seed sold as a cheap blended product. Recent attempts to market newly released turf and forage varieties as value-added, proprietary cultivars has not always gone well because of pressure placed by consumers on seed marketers and distributors to lower prices on proprietary cultivars due to the abundance of inexpensive commodity seed. Currently, to now move toward high-cost technology products will surely require a new model for seed production, marketing, and sales, and farmers who purchase such products. It remains to be seen how this can be done successfully. The widespread adaptation of endophyte technology, as the first technology in grass cultivars, could set the tone for the grass seed industry in the future. If commercial endophytes fail to justify their development and marketing costs because of cheap seed and poor quality control, then what initiative will grass seed companies have to invest in any new technology in the future?
5. CONCLUSIONS

Fungal endophytes are found in mutualistic associations with many grass species. The unique property of several of these associations is their ability to impart good persistence and performance into their grass hosts. The endophytes with commercial value are currently in the Neotyphodium genus, with use and development focused on the tall fescue/N. coenophialum and the perennial ryegrass/N. lolii associations. The main successes have been with the many turf cultivars that are sold as “endophyte enhanced,” the successful use with proper management of toxic E+ pasture cultivars such as Kentucky 31 and Georgia 5 tall fescue, and most recently, the successful reinfection of nontoxic endophytes such as Endosafe and MaxQ into elite perennial ryegrass and tall fescue cultivars, respectively. Common technical problems during commercialization of endophytes include screening methods to assess both amount and type of infection, maintaining endophyte viability during seed increase and dissemination, and conducting the requisite agronomic and animal testing before actual release of new cultivars. Finally, endophytes are the first patented technology to be placed in grass cultivars, but the current commodity approach of the grass seed industry seems ill-equipped to deal with intellectual property issues such as protection and high seed prices. If commercial endophytes fail to justify their development and marketing costs, then what initiative will anyone have to invest in any future technology to be carried in a grass cultivar host?

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Use of Clavicipitalean Fungi for the Biological Control of Arthropod Pests

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1. INTRODUCTION

Biological control or biocontrol is a much-abused and frequently misunderstood term or concept. In its simplest, and hence purest form, it is the manipulation of natural enemies by humans to control pests. This is clearly distinguished from natural control, which is omnipresent and occurs without people’s conscious or deliberate interventions (Evans, 1974; Samson et al., 1988). A pest, sensu lato, constitutes any organism which proves to be troublesome to humans, either directly or indirectly, in a particular country, ecosystem, or habitat. Thus, even the most useful species can become highly invasive and achieve pest status in exotic situations, as for example *Pinus* and *Eucalyptus* spp. in South Africa (Cronk and Fuller, 1995; Henderson, 1998).

Such pest species are termed immigrants or aliens, and a tried and tested management strategy for these invasive exotics is to introduce their coevolved natural enemies, which by association, and of necessity, must be specific to the target pest species or its genus. This is classical or inoculative biological control, and adheres to a well-regulated “Code of Conduct” for the importation and release of exotic agents (FAO, 1996). Typically, this approach using fungal
biocontrol agents is sustainable or self-perpetuating and has been employed almost exclusively against invasive alien weeds (Evans and Ellison, 1990; Evans, 2000, 2002; Evans et al., 2001). It should not be confused with the misguided (nonscientific) but oft-quoted attempts to control alien insects and higher animals with imported, nonselective or polyphagous predators, much beloved by television documentary makers and even popular scientific journals because of the catastrophic but entirely predictable negative impacts that such generalist feeders have on the “unfortunate” ecosystems into which they have been introduced.

The other approach, termed inundative or augmentative biological control, involves the mass production of an indigenous natural enemy, which can have either a narrow or a wide host range, and its periodic application to the pest target. Most of the literature and activities pertaining to the biological control of arthropod pests with fungi of clavicipitalean origin is concerned with this strategy, and with the development of formulated, semicommercial or commercial products: the so-called mycopesticides or mycoinsecticides (Samson et al., 1988). However, the distinctions between the inundative and classical approaches are blurred in the case of fungal biocontrol agents of arthropods, especially compared to those of weeds, and there are few examples where the classical strategy has deliberately been adopted. So far, these have all involved fungi belonging to the Entomophthorales, the most recent being the introduction of an Australian pathotype of *Entomophaga grylli* (Fres.) Batko into the United States for control of grasshopper pests in rangeland (Carruthers and Onsager, 1993). This followed on from a highly successful program in Australia in which an aggressive pathotype of *Zoophthora radicans* (Bref.) Batko from Israel was released to control an exotic aphid species of alfalfa crops (Milner et al., 1982; Carruthers and Hural, 1990). Other possible “classical” projects are still in the pipeline, and studies have been undertaken recently in Brazil on a coevolved entomopathogen, near *Neozygites floridana* (Weiser & Muma) Remaudiere & Keller, of the cassava green mite in order to determine its potential as a classical biocontrol agent of this exotic pest in West Africa (Odour, 1995).

Nonetheless, there are examples in which the classical approach has been indiscriminately or nonconsciously used with clavicipitalean fungi whereby commercial mycoinsecticides, and even crudely formulated, cottage-industry products based on their anamorphs, have been and still are being moved routinely around the world for testing against selected target pests, apparently without legislative requirements. In essence, of course, although these may be based on indigenous, cosmopolitan species, the strains involved are exotic and imported without pest risk assessments (Hajek et al., 2001). However, only recently have nontarget effects been taken into account and evaluated critically for such products (Hajek and Goettel, 2000; Goettel et al., 2001). This is in sharp contrast to weed pathogens, for which an aura of pathophobia invariably surrounds their
international movement as classical biocontrol agents (Evans and Ellison, 1990; Evans, 2000), and which demands strict guidelines and strongly enforced quarantine procedures (FAO, 1996).

Because of the difficulties and inconsistencies in categorizing the past and present uses of clavicipitalean fungi for the control of arthropod pests, this chapter will approach the subject not from the strategies adopted but by analyzing the individual genera involved. As suspected, all of the Deuteromycetes which have traditionally been exploited as arthropod biocontrol agents have now been proven to belong to the Clavicipitales, using both cultural and molecular techniques. Thus, the chapter is arranged logically, initially reviewing uses of the teleomorph, followed by the anamorph genera in alphabetical order.

2. THE GENERA INVOLVED

2.1. Cordyceps (Fr.) Link

There are only two authenticated examples in which the actual Cordyceps fructifications or ascospores have been incorporated into a management strategy for control of arthropod pests.

The first involves a Cordyceps sp., close to C. typhulaeformis Berk. & Cooke, which periodically causes epizootics in both the larvae and pupae of nettle caterpillars (Limacodidae) in Malaysia and Indonesia (Evans, 1987). Limacodids are important pests of plantation tree crops, especially of palms in Southeast Asia (Wood, 1987), and attempts to control them by exploiting entomopathogenic fungi in general, and Cordyceps in particular, have been ongoing since the 1940s. Schneider (1940) reported Cordyceps outbreaks on limacodid cocoons in Indonesia, describing the causal species as having “carmine red fruiting bodies.” Attempts were made to increase infection levels by applying crude suspensions, containing macerated material of both the mummified host and the Cordyceps stromata, to healthy pupae; as well as exposing them directly to diseased specimens. Infectivity of this inoculum—presumably containing a mixture of fungal mycelium, chlamydospores and ascospores—was proven, although disease levels were relatively low (13–33%). Similar methods were employed some 30 years later in Malaysia, when aqueous suspensions of diseased pupae were sprayed around tree bases, the favored pupation sites of limacodids. High infection rates were reported, particularly during the rainy season (Mackenzie, 1977). Subsequently, Tiong (1979, 1982) improved the purity, formulation, and application strategy of this “product” during field trials against limacodid pests of oil palm and coconuts in Sarawak. Field-collected, recently killed pupae were held in soil beds at high humidity until completion of stromatal development (ca. 35–40 days). Mature fruit bodies, identified by the “honeydew” appearance imparted by the exuded ascospores, were triturated and mixed with
distilled water (5 stromata/L). The resultant spore suspensions were sprayed around the tree bases in a 20-cm-wide band at the rate of 120 mL per tree. The pathogen gradually established itself within the limacodid populations and built up to epizootic proportions over a 12-month period, when infection rates of 80–100% were recorded. It was concluded that application of the fungus alone was sufficient to keep the limacodid populations below the economic pest threshold level without the need for other treatments, such as chemical insecticides. As with many of these cottage-industry—or prototype—mycoinsecticides, there appears to have been no investment or follow-up to mass produce or commercialize a product based on this fungus. Obviously, the use of ascospores has serious limitations and there is a need to “work through” the anamorph which is suspected to be a readily culturable species of *Paecilomyces* or *Verticillium sensu lato*, since these are the representative anamorphic genera of the *C. militaris* group (subsection *pseudo-immersae*, Kobayasi, 1941), to which the limacodid fungus pertains (Evans, 1987; Samson et al., 1988).

This *Cordyceps* sp. is almost certainly a coevolved natural enemy of Limacodidae, and there are consistent records of a *C. militaris*-type species on these hosts from Asia (Evans, 1987), which only becomes prominent during pest outbreaks and probably appears too late to prevent economic damage to the crop. Thus, the rationale of introducing the pathogen in quantity early in the wet season in order to “kick-start” or induce premature epizootics would appear to be a cheap and potentially effective way of preventing population explosions of the pest and, perhaps, of maintaining these populations at subeconomic levels. The *Cordyceps* could even be used in conjunction with low-dose chemical insecticides in order to improve efficacy, since there is evidence from Sabah of a synergistic association between this pathogen and conventional insecticides, when epizootics of *Cordyceps* were linked to prior applications of insecticides such as lead arsenate (Wood and Nesbit, 1969).

As a taxonomic precaution, the identity of this *Cordyceps* sp. (Fig. 1) is still left open to doubt, since limacodid hosts are variously deposited in both Herb. IMI and Herb. K under the names *C. coccinea* Penz. & Sacc., *C. militaris* (Fr.) Link, *C. pruinosa* Petch, and *C. typhulaeformis* (Petch, 1942; Evans, 1987). Clearly, there is a need for a taxonomic reevaluation of the pathogens associated with limacodid hosts and of the actual role that they play in pest population dynamics, as well as of their potential value as biological control agents of these pest species in Asia.

The second example concerns a *Cordyceps* pathogen of melolonthid beetles (Scarabaeidae) in both Africa and Asia, where the larval stages or white grubs are important constraints to sugarcane cultivation. This *Cordyceps* sp., currently assigned to *C. barnesii* Thwaites (Figs. 2 and 3), regularly induces epizootics amongst white grub pests in sugarcane plantations in East Africa and Indonesia (Hocking, 1966; Evans et al., 1999). In the latter country, plantation
FIGURE 1 Cordyceps sp. on Limacodid (nettlegrub) pupa, oil palm soil, Malaysia. Note that the larval case with irritating hairs has been removed to reveal the white mycelium covering the pupa. The cylindrical, *C. militaris*-like stromata or clavae are carmine red when fresh.
**Figure 2** Longitudinal section of dark brown, clavate stroma of *Cordyceps barnesii* showing the perithecial chambers and remnants of the *Hirsutella* anamorph.

**Figure 3** Ascus tips of *C. barnesii* showing the central pore, typical of *Clavicipitales*.
workers actively deploy *Cordyceps*-infected larvae within and between plots to create new infection foci and thus artificially increase disease levels. A *Hirsutella* anamorph (see Fig. 2) is produced in culture from ascospores, or from resting bodies taken from the mummified abdomen, but it is very slow-growing and would not be amenable to mass production. Consequently, there are serious doubts about the potential of this species as a commercial or even a semicommercial, cottage-industry-type product for control of sugarcane white grubs. What is certain is that sugarcane managers are sufficiently aware of its importance as a periodic, natural control agent to have adopted a “low-tech” method of inducing premature epizootics. Such simple manipulation techniques can constitute a feasible management strategy in the sugarcane agroecosystem, where, traditionally, there is an abundance of cheap labor and an efficient infrastructure in place in order to implement it on a large scale.

Of the other clavicipitalean genera, no records can be found concerning the use of the teleomorph per se as an infective unit for the biological control of arthropod pests. However, the literature on the exploitation of their anamorphs is vast and predates that on chemical pest control. Thus, it is not feasible to encompass all of this information in a single chapter. Instead, selected examples are presented for each anamorph genus to reflect both the past history and the current situation.

### 2.2. *Aschersonia* Mont.

For many years, *Aschersonia* spp. (Fig. 4) and their *Hypocrella* teleomorphs (Fig. 5) were considered to be leaf parasites, and it was not until the 1890s, some 50 years after the initial erection of the genus, that Webber (1897) reported on their true entomopathogenic nature, specifically as important natural control agents of citrus pests in the United States. Nevertheless, this supposed plant association persisted, as evidenced by the views of Parkin (1906), who reported on their occurrence in the forests of Sri Lanka: “Quite possibly in some of these cases a scale insect is the real host and not the plant.” However, Parkin (1906) did review the attempts of U.S. Department of Agriculture (USDA) scientists to employ *Aschersonia* spp., along with other entomopathogens (the so-called friendly fungi), as biocontrol agents of the citrus whitefly, *Dialeurodes (Aleyrodes) citri*, in Florida. Later, this work was summarized by Rolfs and Fawcett (1908), who even listed the suppliers from whom fungal isolates and fungal-infected materials could be purchased by the growers. Among these “products” were the “yellow and the red fungus of the whitefly” or *Aschersonia flavocitrina* P. Henn. (= *A. goldiana* Sacc. & Ellis) and *A. aleyrodis* Webber, respectively. Different application strategies are described in detail, including pinning citrus leaves bearing *Aschersonia*-infected whiteflies in the canopy of
pest-infected trees at the beginning of the rainy season, and spraying crude conidial suspensions onto the undersides of leaves using compressed-air sprayers.

However, the efficacy of such treatment was later brought into question by Morrill and Back (1912), who concluded that: “All experiments have shown that it is useless to force nature and that fungi cannot be successfully introduced unless the weather conditions are such that the fungi are spreading naturally in infected groves.” Petch (1925), in his comprehensive but pessimistic treatise on “Entomogenous fungi and their use in controlling insect pests,” concurred with these findings. Nevertheless, despite this negative report, a semicommercial product was developed and sold to citrus farmers by the Florida Plant Board, the *Aschersonia* spp. being grown on sweet potato strips in wide-mouthed pint bottles (Berger, 1921). With the advent of chemical insecticides in the 1920s, interest in the exploitation of entomopathogenic fungi for whitefly control lapsed. Within the past 10–20 years, however, there is evidence of renewed interest in whitefly pathogens, as pest resistance to chemical insecticides has gained ascendancy. Samson and Rombach (1985) reported on the use of *Aschersonia aleyrodis* in Eastern Europe to control the greenhouse whitefly, *Trialeurodes vaporariorum*. These authors also reported on experiments in the Netherlands to control this greenhouse pest and found that exceptionally high doses (10^{13} conidia/hectare) of *A. aleyrodis* were necessary to achieve complete kill in a cucumber crop. Quinlan

**Figure 4.** *Aschersonia* conidia germinating in vitro to produce a secondary spore stage (see Evans and Hywel-Jones, 1997).
(1988) also assessed the biological potential of *A. aleyrodis* against greenhouse whitefly pests, based mainly on in-house research undertaken by the Dutch biocontrol company Koppert B.V., and concluded that although this pathogen is highly infective, and probably faster-acting compared to other fungal biocontrol agents, it is expensive to produce and only attacks the larval stages (Fransen et al., 1987). Moreover, it is difficult to manipulate within the greenhouse system in order to achieve consistent and, most important, sustainable control.

Evans (1988) and Evans and Hywel-Jones (1997) remained optimistic about future exploitation of this genus since *Aschersonia* spp. are ubiquitous and important natural enemies of both Aleyrodiidae and Coccidae, having coevolved with them in tropical forest ecosystems (Fig. 6). Undoubtedly, such habitats constitute a potential but threatened source of new species and isolates which could be used not only in the long-term management of these increasingly problematic agricultural pests, but also as leads to novel metabolic pathways. However, more investment in basic research on their taxonomy and biology, as well as on formulation technology, is needed if this potential is ever to be realized.
FIGURE 6  Original water color illustration from Petch (1921), showing the
diversity of *Hypocrella–Aschersonia* spp in the forests of Ceylon (Sri Lanka).
2.3. **Beauveria Vuill.**

*Beauveria* has long been considered to be a clavicipitalean anamorph (Arx, 1986; Samson et al., 1988). However, irrefutable proof was obtained only when Shimazu et al., (1998) described a new *Cordyceps* species (*C. brongniartii*), ascospore isolations from which produced *Beauveria brongniartii* (Sacc.) Petch in culture.

“Enough to fill volumes has been written about the silkworm disease, or Muscardine, which it is by no means our intention to reproduce here” wrote Cooke (1892) about *Beauveria [Botrytis] bassiana* (Bals.) Vuill. at a time when it was viewed, in mycological circles at least, as a pest rather than a beneficial species. Coincidentally, however, around this period, scientists in the United States were pioneering methods to utilize this fungus, then identified as *Sporotrichum globuliferum* Speg., for the control of the chinch bug (*Blissus leucopterus*), a serious pest of grain crops in the U.S. Midwest (Petch, 1925; Steinhaus, 1949, Samson et al., 1988). Initially, diseased insects were distributed free to growers in Kansas from a central experiment station. According to Petch (1925), between 40,000 and 50,000 "packages" were produced over the 6-year campaign (1891–1897). As with the “friendly fungi” in Florida, an independent state review board was convened to evaluate the control program and a similar damning report resulted: “Advocating artificial infection or encouraging it by sending out diseased chinch bugs does not serve the best interests of the farmer, since his attention is thus diverted from other, more efficient methods of combating the pests” (Billings and Glenn, 1911). Tons of cottage-industry and commercially produced conidia of *B. bassiana* have blown about in the air since this report; for example, it was estimated that over 20 tons of the registered mycoinsecticide Boverin were being manufactured per annum in the 1970s (Roberts and Yendol, 1981). Nevertheless, as with many mycopesticides, it is difficult to determine the current status of the numerous semicommercial and commercial products based on *B. bassiana* which have been reported in the literature in both past and recent times. Suffice it to say that this entomopathogen has been and still is being evaluated and targeted against a range of arthropod pests worldwide, including the Andean potato weevil (*Premnotrypes latithorax*) in Peru; the coffee berry borer (*Hypothenemus hampei*) in Colombia; the Colorado beetle (*Leptinotarsa decemlineata*) in Europe; the cotton boll weevil (*Anthonomus grandis*) and whitefly (*Bemisia tabaci*) in the United States; the Sunn pest (*Eurygaster spp.*) in the Middle East; the cabbage looper (*Plutella xylostella*) in Malaysia; the pine Sawyer (*Monochamus alternatus*) in Japan; and the pine caterpillar (*Dendrolimus punctatus*) and the European corn borer (*Ostrinia nubalis*) in China. In the latter example, Hussey and Tinsley (1981) estimated that over 400,000 hectares were being treated annually, the inoculum being produced under relatively primitive conditions in communes, with more
than 1000 of these units in operation by the end of the 1970s in the Yangtse
Valley alone, where, over a 5-year campaign, corn borer damage was reduced
from 60% to 2%.

The history of attempts to exploit *B. bassiana* for pest control is long,
confused, often conflicting and bizarre—as, for example, employing landmines
and mortar shells to propel inoculum into forest canopies in China—and can be
traced though diverse sources (Petch, 1925; Schaefer, 1936; Steinhaus, 1949;
Dunn and Mechalas, 1963; Ferron, 1981; Gillespie, 1988; Wright and Chandler,
1992; Feng et al., 1994). Current information indicates that, in addition to
Boverin, there are three registered products on the market in the United States
based on *B. bassiana*: Mycotrol, BotaniGard, and Naturalis (Troy BioSciences)
(Wraight and Carruthers, 1999). Mycotech operates a commercial-scale
production facility for the first two products, which are wettable powders.
Mycotrol functions well in the field against a broad range of pests, while
BotaniGard is for greenhouse use, specifically against aphids, thrips, and
whiteflies. In addition, Butt et al. (2001) list other mycoinsecticides which are in
various stages of development: Conidia (Live Systems Technology, Colombia);
Ostrinil (Natural Plant Protection, France); and Proecol (Probioagro, Venezuela).

*Beauveria brongniartii* has an equally long and chequered history as a
biocontrol agent, particularly of melolonthid or cockchafer pests, and large-scale
trials of this fungus (then known as *B. tenella* Sacc.) were in operation at the end
of the nineteenth century in France. This early work is reported upon by Ferron
(1978, 1981), who reinvestigated this pest–pathogen complex, specifically in
pasture systems in eastern France, and who experimented with novel liquid
injection methods to deliver blastospore inoculum (at $2 \times 10^{13}$ spores/ha) directly
into the soil. Prolonged and chronic infection of the chafer larvae, which later led
to epizootics, was demonstrated, and the adults were shown to vector the disease
into the next generation. Keller (1986) built on these promising results for
integrated management of the May beetle or European cockchafer (*Melolontha
melolontha*) in Switzerland. Spores, at the rate of $2.6 \times 10^{14}$/ha, were applied
both to the larvae, using tractor-mounted spray booms, and to the adults
congregating at the forest margins, by helicopter. Although costs were high, the
methodology was considered to be economically viable since it gave ongoing
control—populations typically crashed during the second year—and, by targeting
the swarming beetles before they dispersed, several thousand hectares of pasture
and crops could be protected.

The success of this work led to the registration of the pathogen in 1990 for
use against cockchafer pests in Europe, which spawned a number of products
(Keller et al., 1997; Butt et al., 1999; Inglis et al., 2001): Engerlingspilz
(Andermatt, Switzerland); Schweizer (E. Schweizer, Switzerland); Melocont
(Kwizda, Austria); and Betel (Calliope, France). However, the current market size,
and hence the commercial viability, of these products is difficult to determine.
2.4. *Hirsutella* Pat.

The genus *Hirsutella* comprises a somewhat heterogeneous mix of species broadly divided into two sections, mononematous and synnematous (Minter and Brady, 1980). The former grow relatively well in vitro, and all represent *Torrubiella* anamorphs, while the true synnematous species are often difficult to establish in culture and, typically, the anamorph represents an early stage in the development of the *Cordyceps* stroma or clava (Evans and Samson, 1982a, 1984). Ascospores of some species can even germinate through microcyclic conidiation to produce *Hirsutella* conidiogenous structures directly (Samson et al., 1982).

*Hirsutella thompsonii* Fisher (Fig. 7) was not formally identified and described until many years after its role as an important pathogen of the citrus rust mite (*Phyllocoptruta oleivora*) was first suspected (Speare and Yothers, 1924; Fisher, 1950). Undoubtedly, the species was an unrecognized component of the “friendly fungi” of Florida citrus farms, discussed earlier. However, it was not until relatively recently that its actual impact on pest populations was quantified and its commercial potential as a biocontrol agent of both eriophyid and tetranychid mites was realized (McCoy, 1981). This resulted in the launch of a commercial mycoinsecticide, Mycar, for early application in citrus orchards to

![Figure 7](image_url)  
*Hirsutella thompsonii* from in-vitro culture, isolated from a mite host.
promote epizootics in mite populations (McCoy, 1986). Although *H. thompsonii* is apparently still registered as a microbial control agent of citrus rust mite in the United States (Tanada and Kaya, 1993), Mycar is not included in an updated list of U.S. registered mycoinsecticides (Wraight and Carruthers, 1999), which may, in part, be due to its complicated and hence expensive two-step production system, coupled with inconsistent field performance (McCoy, 1986). The potential of *H. thompsonii* as a biocontrol agent of other eriophyid mite pests, such as the coconut mite in the Caribbean (Moore et al., 1989), has been and still is being evaluated but, thus far, without any indications that it has actually been realized, commercially or otherwise.

Rombach et al. (1989) later reported on a much-improved methodology for cheap and efficient mass production of *Hirsutella* inoculum, involving the use of dry mycelial preparations produced by the marcescent process. Major homopteran pests of both rice (*brown planthopper, Nilaparvata lugens*) and fruit trees (*mango leafhopper, Idioscorpus clypealis*), were targeted in the Philippines with specific *Hirsutella* pathogens, *H. citriformis* Speare and *H. versicolor* Petch, respectively. However, there are no further reports of commercial exploitation of these species, nor of registered products based on them.

Similarly, a recently described *Hirsutella* species, *H. cryptosclerotium* Fernández-García et al., associated with outbreaks of an exotic mealybug pest (*Rastrococcus invadens*) of fruit trees in West Africa (Fernández-García et al., 1990), was evaluated as a biocontrol agent. A proto-mycoinsecticide was developed for field trials in Togo, based on the marcescent process (Fernández-García, 1990), but these were subsequently postponed indefinitely when the pest was shown to have been brought under control following the introduction of a coevolved hymenopteran parasitoid (*Gyranusoidea tebygi*) from Asia (Agricola et al., 1989), and “must surely rank as one of the most successful biocontrol operations in recent years” (Williams, 1989). This clearly demonstrates both the benefits of a holistic, integrated control strategy and of the tremendous but largely untapped potential of the classical biological control approach to pest management.

2.5. *Metarhizium* Sorokin

Seifert and Gams (2001) considered that the teleomorph of *Metarhizium* is still unknown. However, the connection with *Cordyceps* was firmly established by Zong-Qi et al. (1991), after they reported microcyclic conidiation in the ascospores and described the new species *Metarhizium taii*. Thus, the long-held view that *Metarhizium* represents an anamorphic clavicipitalean genus has now been confirmed.
Petch (1925) detailed the early history of the green muscardine fungus, *Metarhizium anisopliae* (Metsch.) Sorokin, and described some of the early attempts in Russia over a century ago to exploit this entomopathogen for pest control. Coincidentally, this occurred at about the same time that *Beauveria bassiana* was being field-tested in the United States, and both of these pioneering examples laid the foundations for insect pathology in general, and mycopesticides in particular. Although these early efforts were seemingly unsuccessful and much criticized, more positive reports subsequently emanated from Trinidad, where a coevolved strain of *M. anisopliae* was employed over a number of years against the sugarcane froghopper, *Aenealalimia varia saccharina* (Rorer, 1913). Petch (1925), and more recently Allard (1987), have analyzed the relatively sophisticated mass-production techniques and application strategies involved, as well as the results obtained by Rorer and co-workers over a 10 to 15-year period. The conclusion reached, however, was that “the use of the green muscardine becomes a form of gamble” (Williams, 1921), and “the artificial distribution of spores does not appear to have made any appreciable difference to the froghopper blight” (Petch, 1925). Nevertheless, the jury is still out on the potential of *M. anisopliae* as a biocontrol agent of this important pest, and renewed investment is being made in research and development, following the conclusion of Allard (1987) that “conditions appear favorable in Trinidad for augmentative release of the indigenous strain of *Metarhizium*.” A similar pathogen–pest association occurs in the sugarcane regions of Brazil, and a semicommercial product, Metaquino, has been reported to be currently in use (Butt and Copping, 2000).

The many crop–pest complexes in which *M. anisopliae* has been identified as a primary component, and the equally numerous attempts to exploit the fungus, have been well documented (Ferron, 1981; Bartlett and Jaronski, 1988; Gillespie, 1988; Inglis et al., 2001), and will not be discussed further. Instead, the aim here is to draw attention to recent significant developments.

The Bio-Path Cockroach Control Chamber (EcoScience, USA) is promoted for its ecological or green credentials, using “nature to control nature’s pests” (Andis, 1994). In essence, it employs a bait technique to lure cockroaches inside, and the chamber design ensures that, as they leave, they are inoculated with dry conidia of *M. anisopliae*. Horizontal transmission within the insect populations is an integral component of this strategy (Kaakeh et al., 1996).

From these relatively small niche markets, *M. anisopliae* is also being developed for large-scale use against grasshopper and locusts pests worldwide. Although entomopathogenic fungi have been known to be key mortality factors in the acridids for many years (Petch, 1925; Schaefer, 1936), chemical control has dominated the management philosophy until recently. However, environmental concerns have led to the reduced use or outright banning of many anti-locust pesticides, particularly the organochlorines and organophosphates, such as
dieldrin and malathion, respectively (Murphy et al., 1994; Lomer et al., 2001). A 10-year program was initiated in 1989 to evaluate the entomopathogenic mycobiotai associated with acridids and to develop an appropriate mycoinsecticide. This has resulted in a registered product Green Muscle, based on an acridid-specific strain of *Metarhizium* and applied in an oil formulation at ultra-low volume (Bateman, 1997; Lomer et al., 1997). Until recently, this has been considered to belong to *M. flavoviride* W. Gams & Rozsypal, but molecular evidence has now placed the pathogen in *M. anisopliae* as a distinct variety, var. *acridum* (Driver et al., 2000). The mycoinsecticide, which kills up to 90% of treated locusts with no negative impacts on nontarget organisms, is under licensed production in South Africa, and is also being trialed against both locust and grasshopper pests in Europe and the Americas.

During the project, a range of entomopathogenic fungi was collected from around the tropics, including a “*Verticillium*” sp. (Fig. 8) derived from the ascospores of *Cordyceps locustophila* P. Henn., a pathogen of forest locusts in the Neotropics. A number of *Cordyceps* spp. are associated with acridid hosts in

**Figure 8** Un-named “*Verticillium*”—type anamorph isolated from *Cordyceps* sp. in the Brazilian Amazon.
South America but, significantly, no *Cordyceps* specimens were recorded on locusts or grasshoppers during the intensive, 3-year collecting period in Africa (Shah et al., 1997).

Another host-specific variant of *M. anisopliae*, var. *majus* (Johnston) Tulloch, a coevolved pathogen of rhinoceros beetle (*Oryctes rhinoceros*), has long been associated with epizootics in beetle populations and recognized as an important natural control factor in the Philippines (Bedford, 1980; Young, 1986). However, changing agricultural practices, such as the introduction of coconut dwarf varieties and the wholesale poisoning of existing plantations, as well as natural disasters (typhoons), have contributed to massive and damaging beetle outbreaks and the loss of control from its natural enemies. To counteract this, control efforts have concentrated on augmenting beetle pathogens, both the fungus as well as a baculovirus, and applying them through a farmer-participatory approach (Young, 1986; H. C. Evans, unpublished data). Cottage-industry-type fungal inoculum—produced on rice grains in flat, rum bottles—was distributed to farmers who prepared aqueous suspensions containing a sticker, in which field-collected beetle larvae were immersed and the inoculated grubs subsequently disseminated around the farm, preferably in the dead standing or fallen palms. Efficient horizontal dispersal within these breeding sites was demonstrated. However, there is no evidence that this cheap, low-tech approach was ever taken up on a large scale.

Apart from the products included above, a number of other *M. anisopliae* -based mycoinsecticides are currently registered for use against arthropod pests (Butt et al., 2001): BIO 1020, against vine weevil (Bayer, Germany); BioBlast for termite control (EcoScience, USA); Biogreen used against chafers (Bio-care Technology, Australia), and, Cobican to combat spittlebugs (Probioagro, Venezuela).


Evans (1982) confirmed *Nomuraea* as a clavicipitalean anamorph when *N. atypicola* (Yasuda) Samson was obtained from ascospore isolations of *Cordyceps cylindrica* Petch, a pathogen of trapdoor spiders in neotropical forests. Interestingly, the anamorph has never been found on the host in South America, in sharp contrast to the situation in Asia, where *N. atypicola* dominates, producing prominent synnemata reminiscent of the stromata of *C. cylindrica* (Kobayasi, 1941).

*Nomuraea rileyi* (Farlow) Samson has excited interest as a potential biocontrol agent of noctuid pests, particularly of soybean loopers, for many years (Getzin, 1961): “In Puerto Rico it is possible that its usefulness could be increased in times of severe invasions of the common grass worm by artificial distribution” (Johnston, 1915). Epizootics are common but occur too late in the
season to prevent economic losses (Fuxa, 1984). Thus, the control strategy in the United States, which is based on mathematical modeling of natural epizootics, has been to introduce inoculum into the crop system early in the season as foliar sprays (Ignoffo, 1981). Despite the initial optimism, this potential has not been realized and no product has yet reached the registration or marketing stage (Wraight and Carruthers, 1999). Inconsistent field performance, allied to difficulties in mass producing the fungus cheaply and efficiently, were probably contributory to the apparent commercial failure of N. rileyi. However, recent reports indicate that application to the soil, rather than to the foliage, holds promise as an alternative pest control strategy (Devi, 1995).

2.7. *Paecilomyces Bainier*

It has proven difficult to confirm the oft-repeated assumption that *Paecilomyces* is a *Cordyceps* anamorph (Samson et al., 1988), due to lack of cultural proof. As pointed out by Samson and Evans (1977), “There is no indication that *P. tenuipes* represents the imperfect state of some *Cordyceps* species, as suggested by Petch (1933) and Kobayasi (1941).” However, evidence has now been obtained from a molecular phylogenetic analysis carried out by Fukatsu, et al., (1997), which showed that *Paecilomyces tenuipes* (Peck) Samson belongs to the monophyletic group of the Clavicipitales. Ascospore isolations from several undescribed *Cordyceps* spp. from Amazonia have also yielded *Paecilomyces* type anamorphs in culture (H. C. Evans, unpublished data). (Figs. 9 and 10).

As mentioned previously, whiteflies are becoming increasingly important agricultural pests following the overuse of synthetic insecticides, and alternative solutions are being sourced. *Paecilomyces fumosoroseus* (Wize) Brown & Smith is an important mortality factor of adult whiteflies in both field and greenhouse situations, which also exhibits oviocidal and larvicidal activity (Lacey et al., 1996, 1999). Following successful trials with blastospore formulations of this fungus, two commercial products are currently available in North America for whitefly control (Wraight and Carruthers, 1999; Butt et al., 2001): PFR-97 (Eco-tek or ThermoTrilogy, USA) and Pae-Sin (Agrobionsa, Mexico).

2.8. *Verticillium Nees*

Most of the entomopathogenic species formerly placed in *Verticillium* sect. *Prostrata* W. Gams have recently been transferred to the new genera *Lecanicillium* W. Gams & Zare and *Simplicillium* W. Gams & Zare, based on morphological and molecular data (Gams and Zare, 2001). Further analyses of the nuclear ribosomal DNA showed that the majority of species in the *Prostrata* section are Clavicipitaceae, although they do not form a monophyletic group within the family (Sung et al., 2001). *Cordyceps militaris* (Fr.) Link has a *Lecanicillium* anamorph, while the ubiquitous *Verticillium lecanii*, now known as...
Lecanicillium lecanii (Zimm.) Zare & W. Gams, has a Torrubiella teleomorph, *T. confragosa* Mains (Evans and Samson, 1982b).

The history of the attempts to exploit *Lecanicillium lecanii sensu lato* as a biocontrol agent of arthropod pests goes back almost as far as that of *Beauveria* and *Metarhizium*. Petch (1925) reported that at the turn of the nineteenth century, in-vitro production and distribution of *L. lecanii* inoculum was being practiced in coffee plantations in Java to control green scale (*Coccus viridis*). Suspending leaves, bearing fungal-infected scale insects, within coffee bushes was tried later in Sri Lanka in order to induce epizootics within the green scale populations.

*Figure 9* Un-named *Paecilomyces*—type anamorph isolated from *Cordyceps* sp., Brazilian Amazon.
(Parkin, 1906)—a similar strategy to that employed in Florida with *Aschersonia* spp. Both these approaches were summarily dismissed by Petch (1925) in his review of this early history: “The fungus is so generally distributed that artificial distribution could not make any appreciable difference.” Around the same period, efforts were also underway in the Caribbean to enhance the efficacy of *L. lecanii* for control of a range of scale insect pests of fruit trees, and thus, of the attendant sooty molds. Once again, the favored technique was to attach plant material, with fungus-infected scales, to scale-infested trees (South, 1910). Bovell (1912) noted, subsequently, that it was difficult to find healthy coccids on the inoculated trees, and additional recommendations such as spraying trees with aqueous spore suspensions, were made to further improve control. Nevertheless, despite these promising results, there are no indications that any of these methods were adopted on a large scale or even at the cottage-industry level. Interest was
resuscitated some 20 years later in Brazil, when Viégas (1939) demonstrated the value of *L. lecanii* (“the farmer’s friend”) for control of coffee green scale (Fig. 11). However, as previously, this potential was never harnessed, at least commercially or sustainably, and a further 40 years elapsed before more directed efforts were made to exploit *L. lecanii* against the same target pest. Easwaramoorthy et al, (1978) developed a management strategy to control

**Figure 11** *Lecanicillium lecanii* isolated from coffee green scale, West Indies. Note scanning electron micrographs. ([Figs. 8–11](#)), all at scale shown here = 20 μm.
coffee green scale in India which involved using *L. lecanii*, mass-produced on sorghum grain and sprayed at high volume, in combination with low-dose insecticides, and this integrated approach appears to have been adopted and sustained at least at the plantation level (Jayaraj, 1989).

However, the real impetus to develop *L. lecanii sensu lato* commercially was provided by the rapidly increasing acreages of high-value greenhouse crops in Europe in the 1960–1970s, concomitant with burgeoning pest problems and the appearance of “superbugs” as the pesticide treadmill gained momentum (Hussey and Scopes, 1985). Particularly problematic pests, such as whiteflies and aphids, were targeted for biological control, and host-specific “strains of *V. lecanii*” were selected from natural infections for research and development (Hall, 1982, 1984). Two commercial mycoinsecticides were launched on the market: Mycotal, for whitefly control, now classified as *Lecanicillium muscarium* (Petch) Zare & W. Gams and Vertalec, for use against aphids, now classified as *L. longisporum* (Petch) Zare & W. Gams (Fig. 12). In the last 10–15 years, these products have been through a number of owners and currently reside with Koppert B.V. Mycotal is still among the recommended options for whitefly control, and Koppert recently purchased a rival product, MicroGermin, developed by Christian Hansen BioSystems in Denmark (Butt et al., 2001). Vertalec, however, appears to have been withdrawn from the market as an aphicide, possibly due to problems of efficacy (Quinlan, 1988).

Recent taxonomic analyses, using both molecular and morphological characters, reveal that there are four clades or clusters within the “*V. lecanii*” complex (Sung et al., 2001; Zare and Gams, 2001), and that the long-held concept of host-specific strains of “*V. lecanii*” is no longer valid. The current situation is that four species are now recognized: *Lecanicillium lecanii*, the original coffee green scale fungus, which is probably restricted to soft scales (Lecaniidae or Coccidae); *L. muscarium* (Petch) Zare & W. Gams, which includes the Mycotal strain; *L. longisporum* (Petch) Zare & W. Gams, consistently associated with aphids hosts and to which the Vertalec strain belongs; and, *L. nodulosum* (Petch) Zare & W. Gams, which has a disparate host range and may itself be a species complex since it has two unique ITS patterns (Zare and Gams, 2001).

### 2.9. *Tolypocladium* W. Gams

There is some dispute about the validity of the genus *Tolypocladium*. Arx (1986) regarded it as a synonym of *Beauveria*, while Seifert and Gams (2001) still separate *Tolypocladium* on the basis of its discrete phialides rather than the characteristic zigzag, polyblastic, conidiogenous cells typical of *Beauveria* spp. *Tolypocladium inflatum* W. Gams, the type of the genus, has recently been proven to be an anamorph of *Cordyceps subsessilis* Petch, a pathogen of beetle larvae (Hodge et al., 1996). *Tolypocladium cylindrosporum* W. Gams is a specific
pathogen of mosquitoes which, in small-scale trials using blastospore inoculum produced by deep fermentation, caused up to 95% mortality in larvae of *Aedes*, *Anopheles*, and *Culex* mosquitoes (WHO, 1984; Pant, 1986). However, as Bartlett and Jaronski (1988) concluded, considerably more research is needed before the biological control potential of this species can be harnessed.

**FIGURE 12** Aphids 8 days after spraying with a conidial suspension of *Vertalec*, originally classified as *Verticillium lecanii*, now *Lecanicillium longisporum* (see Zare and Gams, 2001).
3. DISCUSSION

Much, perhaps too much, has been written about the actual and potential use of entomopathogenic fungi, many of which pertain to the Clavicipitaceae, for the biological control of arthropod pests. It is not the intention here, nor is it the appropriate place, to fuel the polemic. The pros and cons of the debate can be assessed elsewhere in recent reviews (Moore and Prior, 1993; Wraight and Carruthers, 1999; Butt et al., 1999, 2001; Hajek et al., 2001).

Suffice it to say that interest has waxed and waned over the past 100–120 years, almost on a 20–30-year cyclical basis: initially, “the emergence of an idea” (Steinhass, 1956), which as Petch (1925) cynically wrote, is “still periodically put forward with no better foundation than the discovery of another fungus on another insect”; then, an all-too-short period of experimentation leading to the launch of an application strategy and/or “product”; followed by an independent, usually damning assessment; and, finally, the fading of both the idea and the “product.” In the early beginnings of scientific pest control, there were no alternatives to natural enemies, and clavicipitalean fungi in particular appeared to be the panacea. The optimism and efforts were usually both ephemeral and amateurish, soon to be replaced by professional synthetic chemists, who have now enjoyed a long reign. Is this coming to an end due to the current environmental feeding frenzy, or is there too much at stake to risk all on the biological control lottery? In reality, the stakes have never been high when compared to those placed by agrochemical companies: around US$100 million to develop and register a new synthetic pesticide (Moore and Prior, 1993).

Perhaps a lesson is to be learned from the Green Muscle story (Dent, 2000), which is that it takes both time (minimum of 10 years) and serious money (at least US$5–10 million) to develop a commercial mycopesticide, and not the crumbs usually thrown to such projects with the expectancy of a quick and cheap fix to a pest problem. The emergence of the idea (Prior and Greathead 1989), the search for and the screening of the most robust or virulent fungal strain (Shah et al., 1997; Bateman et al., 1996), the development of the most appropriate mass production technology (Jenkins et al., 1998; Lomer et al., 1997) coupled with high standards of quality control (Jenkins and Grzywacz, 2000), and, of course, formulating and delivering the product to maximize field performance (Bateman, 1997), have all been essential chapters in the story.

Is this the model to follow and will industry invest in it, or simply sit on the fence to await developments as is the norm? Should the much cheaper option of classical biological control, so successfully exploited for weed pathogens, be more closely pursued using entomopathogens for the management of alien arthropod pests? Paradoxically, however, in a world seemingly concerned with biodiversity and environmental issues, securing meaningful funding for such projects, either from the public or the private sector, is becoming increasingly more difficult.
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Evidence for Nematode Defense in Symbiotic Grasses

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1. INTRODUCTION
Grasses are well known for the lack of an abundance of poisonous secondary metabolites that would serve the function of antitherbivory and protection from pests, which provides persistence in a stressful environment within seasonal changes and an ecosystem highly manipulated by human activity. One evolutionary strategy considered to have developed from the lack of diversity of poisonous metabolites was the establishment of a quasi-symbiotic relationship with grazing animals, which led to exploitation of an open habitat, a feat that established grasses as the largest and most dominant plant family. During this period of colonizing an open habitat, grasses developed several morphological, physiological adaptations and ecological associations that collectively accounted for their continued widespread dominance under grazing pressures, which
allowed for the expansion of the family. As a consequence of surviving grazing pressures without development of poisonous secondary metabolites, grasses developed unique morphological adaptations including dense adventitious roots, a strategically placed and highly regenerative basal or intercalary meristem, as well as growth stimulation resulting from grazing or defoliation that is maintained by a unique physiology. In the absence of poisonous secondary metabolites, grasses entered ecological associations with fungi, a group of microorganisms known for the ability to produce numerous and structurally unrelated secondary metabolites, toxic and nontoxic, which provided specialized, ecologically relevant long-term competitive strategies, well beyond the capacity of grasses. One group of fungi, which cohabitated with grasses, perhaps as strict pathogens, is species of the Clavicipitaceae (Ascomycotina). These fungi are endophytic associations that are common to grasses, both temperate and tropical species, sedges and rushes.

The overall results of an endophytic association are the in planta production of unique groups of defensive metabolites, suitable for protecting gasses from vertebrate and invertebrate herbivory, thus controlling or greatly reducing grazing pressures on infected grasses, assuring success in the exploitation of an open habitat. Attention to this line of thinking developed from studies of one temperate grass species, tall fescue (Festuca arundinacea), symbiotic with the fungal endophyte Neotyphodium coenophialum (= Acremonium coenophialum), which exhibited a wide range of protective and fitness enhancements relative to endophyte-free conspecifics. The widespread association of this grass species with endophytic fungi led to the importance of endophytic infections and to the revelation of the several positive ecological benefits associated with infected grasses. Included among these are the pronounced and well-documented accounts of increased osmotic and stomatal resistance, reduced transpiration, resistance or tolerance to pests, increased drought tolerance, and inter- and intrageneric competitiveness (Bacon, 1993; Cheplick et al., 1988; Latch, 1993; Clay, 1988, 1990; Clay et al., 1993; West et al., 1990, 1993; Saikkonen et al., 1996).

Symbiotic grasses are also considered to be resistant or tolerant to nematodes, but early data demonstrating that these pests are indeed controlled by grass endophytes are either incomplete, observational, conflicting, or the endophyte status is unknown. The term “resistant” or “tolerant” as used in this review encompasses the highly modified meaning of these terms, intended as synonyms, in which resistance is not complete in nematode–plant interactions, but is used to describe a reduced level of plant association or lower nematode reproduction on the plant (Trudgill, 1991). At these levels of infections, plant growth and yield are not affected. However, nematodes are very important limiting pests to persistence and yield in grass and range systems (Cook and Yeates, 1993; Bernard et al., 1998). The resulting decrease in production by these pests is intensified when abiotic and physiological factors limiting. Thus, soil
type, water status, plant host resistance and nematode virulence, and population densities are all interacting factors that can affect the final outcome of an infection. This complex interaction makes it difficult to evaluate the effects of nematodes on symbiotic grasses. Since most experiments are also based on the natural situation, the contribution of the many hundred soil nematophagous fungi (Kerry, 1990) cannot be distinguished from controls by a specific endophyte. Nevertheless, most of the data are highly suggestive that there is a direct relationship between nematode reduction in soils and the endophyte infection status of plants, but data concerning resistance or tolerance to fungal diseases are limited and still debated. The evidence for control of plant pathogenic nematodes is strengthened when we consider numerous reports of nematodes being controlled by other fungal and bacterial endophytes, often as biological controls (Sikora and Carter, 1987; Kerry, 1990; Saikkonen et al., 1996; Hallmann et al., 1997; Sturz et al., 1996), and the in-vitro and/or in-vivo production of large potential classes of nematocides, and fungicides by fungal endophytes (Yates, 1983; Porter 1994; Lane et al., 2000).

We review information that nematode control by grass endophytes is a relevant ecological component of endophyte infections, and present some possible chemical compounds that may be responsible for control. The symbiotic grasses discussed in this review include the major species of forage grasses, especially tall fescue, and those species that have been examined specifically to demonstrate antagonisms of symbiotic grasses to nematodes or fungal diseases (Table 1).

2. HISTORIC OBSERVATIONS FOR NEMATODE PREDATION OF GRASSES AND AN ENDOPHYTE CONNECTION

The status of grass infection is particularly important in the case of endophytes, for which most studies were conducted without knowledge of the importance of fungal endophytes. The protective role of tall fescue with *N. coenophialum* was confounded before 1977, the year endophyte status was introduced to science as a cause of animal toxicity (Bacon et al., 1977) and the significant scientific revelation made from this association as a defensive mutualism (Clay, 1988). Historically, very extensive field surveys and greenhouse studies were made without the knowledge of grass endophytes or the effects of nematodes on forage grass species (McGlohon et al., 1961; Hoveland et al., 1975). These studies found nine genera of nematodes in soil samples from 217 forage crops, but that the species of grasses known to be highly infected by fungal endophytes were highly resistant to these nine genera. On the other hand, several species of grasses that are not hosts for endophytes, e.g., Kentucky bluegrass, were highly susceptible to nematodes (McGlohon et al., 1961). These studies are significant in that they
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<tr>
<th>Grass species</th>
<th>Endophyte species</th>
<th>Nematodes species</th>
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<tr>
<td>Perennial ryegrass (Lolium perenne)</td>
<td>Neotyphodium lolii (and other unidentified Neotyphodium species)</td>
<td>Anguina funesta, Meloidogyne naasi, M. marylandi, Pratylenchus scribneri, P. wegluctus, P. penetrans, Heterodera avenae, Helicotylenchus mani, Hel. bifenestra, Tylenchorhynchus dubius, T. maximus, T. claytoni, Puctodera puctata</td>
</tr>
<tr>
<td>Red fescue (Festuca rubra rubra and commutata)</td>
<td>Epichloe festucae</td>
<td>Anguina agrostis, A. funesta, Helicotylenchus digonicus, Hoplolaimus galeatus, Meloidogyne marylandi, M. microtyla, Paratylchlenchus schribneri, Paratylenchus sp.,</td>
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<td>Meadow fescue (Festuca pratensis)</td>
<td>N. uncinatum</td>
<td>Bitylenchus maximus, Helicotylenchus sp., Trichodorus primitivus, Merlinius brevidens, Pratylenchus pratensis, P. goodeyi, P. thornei, Tylenchorhynchus dubius,</td>
</tr>
</tbody>
</table>

*Source:* Data from Cook and Yeates, 1993; Bernard et al., 1998.
present the possible species of nematodes found in an endophyte-infected species, as well as detailing several experimental variables that established tall fescue and other grasses were resistant to nematodes. We now know that these grasses are infected with an endophyte and since there was persistence as evidenced by old pastures, these grasses were infected with \textit{N. coenophialum}, More recently, several ectoparasitic nematodes have been reported to be successfully reproducing parasites or pathogens of tall fescue, although the endophytic status was not considered important (Pedersen and Rodriguez-Kabana, 1984, 1985).

There are several experiments designed to examine relationships of nematodes to symbiotic tall fescue. Densities of \textit{Helicotylenchus dihystera} (Cobb) Sher and \textit{Paratrichodorus minor} (Colbran) Siddiqui were reduced in pastures with high endophyte infestation levels (Pedersen et al., 1988; West et al., 1988). In greenhouse experiments, infection of tall fescue by four species of nematodes was studied. \textit{Helicotylenchus pseudorobustus} (Steiner) Golden, an ectoparasitic nematode, was similar in symbiotic and nonsymbiotic tall fescue (Kimmons et al., 1990). However, the densities of \textit{Tylenchorhynchus acutus} Allen, a migratory ectoparasitic nematode, and of \textit{Pratylenchus seribneri} Steiner was lower on symbiotic tall fescue (West et al., 1988). In spite of these early studies, doubt remains, since Halisky and Myers (1989) still questioned whether the \textit{Neotyphodium} endophyte reduced the densities of soil nematodes. However, proper evaluation of the effects of the interaction of nematodes with grasses depends on nematode species and their density, the nature of feeding types, the nematode stage of development when subjected to specific endophyte substances, and soil interactions. No reports to date, especially field studies, have considered all these variables.

\textbf{3. OCCURRENCE OF NEMATODE SPECIES ON FORAGE GRASSES}

The occurrence and distribution of nematode species established as parasitic on grass species from field accounts or from infections in pot culture are presented in Table 1. These species of nematodes represent different host associations and feeding sites that can be grouped into the mode of parasitism of the nematode species. Thus, there are root-knot, seed gall, and ecto- and endoparasitic migratory root nematodes (Table 2). There are nine additional categories of nematode feeding types, delineated by Yeates (1999) and used to define the wide range of predation practiced by nematodes. In this review we are concerned with only two of the nine major categories of nematodes: plant-associated and plant-feeding types. Plant-feeding nematodes use a stylet to penetrate plant tissue of vascular plants, resulting in yield reduction, and are distinguished from plant-associated nematodes that also feed with a stylet but do not reduce plant yields.
However, these two highly structured categories of feeding types are to be considered with caution, since there is more than one feeding type within a genus and indeed even within a single individual (Yeates, 1999). Again, this indicates the difficulty of assigning conclusive proof as to the role that endophytes play in controlling specific nematodes under field and pasture situations.

There are several lines of evidence that strongly support the argument that symbiotic tall fescue, perennial ryegrass, and other symbiotic species can suppress several nematode species, while others indicate that nematode reproduction may be affected. For example, populations of *Pratylenchus penetrans* (Cobbs) Filip. and Sch. were reduced up to 40% within 8 weeks in soils planted to tall fescue (Townsend et al., 1984). The levels of *Tylenchorhynchus maximus* Allen and *T. ewingi* Hopper were not affected by symbiotic status of tall fescue, but there was a negative interaction with *Paratylenchus projectus* (O’day et al., 1990; West et al., 1988), suggesting that these are degrees of sensitivities to an endophyte product response. Additional greenhouse pot studies using three soil types showed that the migratory endoparasitic nematode *Hoplolaimus galeatus* (Cobb) Throne exhibited more fecundity on tall fescue than on other grasses.
grown in sandy loam, fine sandy loam, or Cecil clay loam (McGlowan et al., 1961; Rodriguez-Kabana et al., 1978), which supports the early observation by Hoveland et al. (1975) that this species is very common in Alabama tall fescue pastures consisting of sandy loam. Thus, soil types are important in dictating which species of nematodes is dominant, which should also be used to describe the nature of nematode parasitism in a field situation.

Kimmons et al. (1990) showed that reproduction of an undescribed root-knot nematode, *Meloidogyne* sp., isolated from white clover was not affected by the endophyte status of tall fescue. This suggests that the ability of symbiotic tall fescue to deter nematodes is specific for fescue-associated nematodes. Information on the interaction of nematodes with tall fescue cultivars suggests that one species of a nematode can parasitize several tall fescue cultivars (Chapman 1973), while McGlohon et al. (1973) found that the parasitic ability was nematode isolate-dependent. Finally, Kimmons et al. (1990) found that *P. schribneri* declined in symbiotic tall fescue, but remained at near initial inoculum levels in non-symbiotic plants. A similar conclusion was reached in a report on *Meloidogyne hapla* Chitwood, *M. marylandi* Jepson and Golden, *M. graminis* (Stedge and Golden) Whithead, and *M. incognita* (Kofoid and White) Chitwood (Kimmons et al., 1990). While these results are somewhat contradictory, they do indicate that there are reductions in nematode densities in soils from symbiotic tall fescue pastures. Specifically, the results suggest that symbiotic tall fescue is at least toxic to sedentary endoparasite and to migratory endoparasitic nematodes. There are no reports of a broad spectrum toxicity effect indicative of specific endophytic toxins known to be produced within the symbiosis.

The susceptibility of plants in general to infection by nematodes varies. Some nematode species do not reproduce on plants, while others of that same species do, and do this regardless of endophyte status. Still, other grass species show strong endophyte-nematode parasitism but no indication of specificity. The possibility of host grass specificity has not been used to account for any difference in data for symbiotic grasses. Some of the data obtained with perennial ryegrass and red fescue suggest that there are host-and nematode-specific interactions within a symbiotic population. This possibility is supported by work on perennial ryegrass in which clonal lines of symbiotic and nonsymbiotic grasses were tested for host effects on *M. marylandi* reproduction (Ball et al., 1997a, 1997b). That is, one endophyte-free clone was as resistant to nematodes as three other infected clones. Other work indicates that egg production on symbiotic plants was 10% lower than that on nonsymbiotic plants (Gwinn and Bernard, 1993). This is important not only from the standpoint of control but also in terms of the type of measurement, i.e., not total nematode numbers, but rather the sex of nematodes and number of eggs produced (Ball et al., 1997a, 1997b). Finally, there is some information to indicate that are genotypes of symbiotic tall
fescues that vary from resistant to susceptible for *M. marylandi* and *M. graminis* (Kirkpatrick et al., 1990; Gwinn and Barnard, 1993; Kimmons et al., 1990; Kirkpatrick et al., 1990; Cook et al., 1991).

Information on the migratory nematodes, the lesion nematodes, and stunt nematodes all seems to indicate that these are indeed controlled by the tall fescue endophyte (Kimmons et al., 1990; Bernard et al., 1998). However, in another field trial in which the nematode level was very low, there was no effect on nematode control (O’Day et al., 1993), indicating to us that there were at least some deterring substances or that the grass can outgrow the nematodes at low levels. Lesion nematodes are apparently not controlled by endophytes in red fescue and perennial ryegrass (Gwinn and Bernard, 1993). However, work from others indicates that endoparasitic populations of *Pratylenchus* species (*P. scribneri* and *P. pratensis*) associated with meadow fescue and perennial ryegrass, grown in both pot and field cultures, were fewer in endophyte-infected plants than in endophyte-free plants (Cook et al., 1991; Schberlein et al., 1997) especially if white clover was also present (Watson et al., 1995). Finally, stunt nematodes research can be summarized by stating that grass variation and endophyte effects are related to nematode control of this species. This conclusion is based on research conducted by West et al. (1988, 1990) on *Tylenchorhynchus acutus*, *T. dubius*, *T. maximus*, and *Merlinius brevidens*. The latter nematode was also reduced by symbiotic *F. pratensis* (Schoberlein et al., 1997). However, there were indications that feeding styles, degree of migration in soil types, and root morphology can alter the effects, since at other locations, there were no effects (Schoberlein et al., 1997; Bridge and Hague, 1974). In this regard, we should be alert to the fact that there are other interacting factors that will prevent us from concluding that there is not a nematode effect. In an excellent analysis, Cook et al. (1991) concluded that while there were more plant nematodes under infected plans, than under noninfected plants, the reason for this difference was related directly to the better growth of endophyte-infected grasses, and this related to the nematode numbers per gram dry weight of roots. Therefore, the actual relationship was reversed: fewer nematodes were found in plots from symbiotic plants.

4. **DEFENSIVE TOXINS AND NEMATODE TOXICITY**

4.1. **Defensive Toxins**

Following is a list of biologically active substances that have been shown to be either derived from the endophyte or found within the association. Particular emphasis is placed on the four known defensive compounds established as effective in being toxic to livestock and insects or instrumental in deterring most pests on endophyte-infected grasses (Table 3). In most instances their
<table>
<thead>
<tr>
<th>Grass species</th>
<th>Endophyte species</th>
<th>Ergot alkaloids</th>
<th>Lolines</th>
<th>Peramine</th>
<th>Lolitrems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrostis hiemalis</td>
<td>Epichloe amerillans</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Dactylis glomerata</td>
<td>E. typhina</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Festuca arundinacea</td>
<td>Neotyphodium coenophailum</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>F. arizonica</td>
<td>N. huefanum</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>F. gigantea</td>
<td>E. festucae</td>
<td>−</td>
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<td>−</td>
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<tr>
<td>F. gigantea</td>
<td>E. festucae</td>
<td>−</td>
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</tr>
<tr>
<td>F. longifolia</td>
<td>E. festucae</td>
<td>−</td>
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<tr>
<td>F. pratensis</td>
<td>N. uncinatum</td>
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</tr>
<tr>
<td>F. rubra</td>
<td>E. festucae</td>
<td>−</td>
<td>−</td>
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<td>−</td>
</tr>
<tr>
<td>Hordeum bogdanii</td>
<td>Neotyphodium sp.</td>
<td>−</td>
<td>−</td>
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</tr>
<tr>
<td>H. brevisubulatum</td>
<td>Neotyphodium sp.</td>
<td>−</td>
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<td>−</td>
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</tr>
<tr>
<td>Lolium perenne</td>
<td>N. lolii</td>
<td>−</td>
<td>−</td>
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</tr>
<tr>
<td>L. persicum</td>
<td>Neotyphodium sp.</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>L. rigidum</td>
<td>Neotyphodium sp.</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>L. temulentum</td>
<td>Neotyphodium sp.</td>
<td>−</td>
<td>−</td>
<td>−</td>
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</tr>
<tr>
<td>Poa ampla</td>
<td>Neotyphodium sp.</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>P. autumanalis</td>
<td>Neotyphodium sp.</td>
<td>−</td>
<td>−</td>
<td>−</td>
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</tr>
<tr>
<td>P. huecu</td>
<td>Neotyphodium sp.</td>
<td>−</td>
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</tr>
<tr>
<td>Lolium perenne</td>
<td>N. lolii</td>
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</tr>
<tr>
<td>L. persicum</td>
<td>Neotyphodium sp.</td>
<td>−</td>
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<td>−</td>
</tr>
<tr>
<td>L. rigidum</td>
<td>Neotyphodium sp.</td>
<td>−</td>
<td>−</td>
<td>−</td>
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</tr>
</tbody>
</table>

Blank, not determined, −, not detected, +, present.
concentrations in symbiotic grasses are increased significantly over nonsymbiotic conspecifics. The exception is the alkaloid perloline, which has been established as present in both symbiotic and nonsymbiotic grasses. In addition to perloline, Gentry et al. (1969) reported that there are as many as 11 different types of identified alkaloids in tall fescue, leading these scientists to the conclusion that this grass is an alkaloid “bearing plant.” Since this declaration, we now know of scores of additional alkaloids in tall fescue. The distribution of alkaloids within other endophyte-infected plants is unknown, but the number in perennial ryegrass is high, and when detailed chemistry is done on it and other infected grasses, the number is expected to be just as significant as tall fescue. The effects of any of these alkaloids, known or unknown, on nematodes have not been established, but as presented below, their potentials as nematode toxins are highly suggestive from studies of broad vertebrate and invertebrate toxicity. Additional studies dealing with fungi as biological control agents for plant parasitic nematodes strengthen this generality.

4.2. The Ergot Alkaloids

Ergot alkaloids were discovered as metabolites of Neotyphodium species in culture, and in planta (Porter et al., 1979; Lyons et al., 1986; Bacon et al., 1988), which established this group of compounds as the cause of livestock toxicity syndromes. The major ergot alkaloid found in Neotyphodium-infected grasses, especially tall fescue, is ergovaline, an ergopeptide ergot alkaloid. This alkaloid and its epimeric pair ergovalinine are also found in symbiotic perennial ryegrass, and in a Hordeum species. Several other ergot alkaloids are also found in most Neotyphodium-infected grasses (Lyons et al., 1986; Siegel et al., 1990; Rowan and Shaw, 1987). The ergot alkaloids are the cause of fescue toxicity, but they are also toxic to insects or deterrents to insect feeding (Siegel et al., 1990; Siegel and Bush, 1996; Ball et al., 1997a; Popay and Rowan, 1994).

4.3. The Loline Alkaloids

The lolines are pyrrolizidine alkaloids, which were initially isolated from tall fescue in 1943 (Cunningham and Clare, 1943). Since this time and after extensive searches, these specific lolines have only been isolated from two other unrelated genera of vascular plants. The alkaloids include the two loline alkaloids (N-acetyl- and N-formylloline, which are two saturated 1-aminopyrrolizidines) (Porter, 1994), collectively referred to as festucine. The production of these two alkaloids by isolated fungal endophytes was only recently demonstrated (Bush et al., 1997). N-acetylnorloline (demethyl-N-acetylloline) is also found in endophyte-infected tall fescue, but is considered an intermediate in the synthesis of the two loline alkaloids, (Porter, 1994; Bush et al., 1993). The concentrations of loline alkaloids increase with plant age, nitrogen fertilization, and foliage
regrowth, and are found in foliage up to 0.1–1.0 % of dry weight, but in root tissue they are found at levels approximately 20% lower (Bush et al., 1993).

The occurrence and concentrations of loline in roots are dependent on soil types. This is another unknown aspect of this association, which might account for the marked variations in the biological response attributed to the loline alkaloids. The biological activity of the two lolines has been suggested as having synergistic potentiating effects with the ergot alkaloids on animal toxicities (Siegel et al., 1991). In addition to being abundant, the lolines lack toxicity to livestock, are highly toxic to a broad range of insect pests (Porter, 1994; Bush et al., 1993; Siegel et al., 1991; Wilkinson et al., 2000), and they are recently being implicated in drought tolerance. The loline alkaloids are used as bioprotectants by other insects that have developed resistance to the lolines, and that cannot make this class of toxins. These insects store these pyrrolizidine alkaloids in their bodies following consumption from plants, and later use these for their own defense mechanisms against other predatory insects (Hartman et al., 1990). They may also be the biologically active factor responsible for the allelopathic properties observed to occur in various plants grown in soils planted with symbiotic tall fescue (Petroski et al., 1990). Thus, the lolines are stable in the soil and biologically active against seed germination and seedling growth. Other alkaloids found in symbiotic tall fescue, include two β-carboline alkaloids, harman and norharman, and an unrelated alkaloid, halostachine (Porter, 1994). Norharman and halostachine have been associated with insect protection and feeding deterrence mechanisms in other forage species (Saunders et al., 1992).

4.4. The Insect Deterrent

Endophyte-infected grasses, especially perennial ryegrass, are noted for resistance to insect predation. The presence of the endophyte in perennial ryegrass offers a tremendous agronomic advantage in terms of insect resistance, such that its effects on cattle toxicity is viewed as a trade-off. The insects affected by symbiotic grasses include the sod webworm, the bluegrass billbug, the southern armyworm, the chinch bug, and the Argentine stem weevil. Recently, perloline was isolated from perennial ryegrass and established as the basis for insect resistance in endophyte-infected grasses (Rowan and Gaynor, 1986; Siegel et al., 1991).

Peramine is a pyrrolopyrazine, and is produced almost universally by symbiotic perennial ryegrass (Rowan and Gaynor, 1986), and in some cultivars and ecotypes of tall fescue, Agrostis hiemalis, and Poa ampla (Table 2). Peramine is found at levels exceeding 10 ppm in perennial ryegrass. It is also found in tall fescue, but its levels and distribution in roots have not been examined, especially in roots. Peramine is highly toxic to invertebrate herbivores (Siegel et al., 1991), especially the Argentine stem weevil. Peramine has antifeeding activity to
several aphids, and it acts synergistic as an insect toxin with other alkaloids (Siegel et al., 1991). Towers (1977) reported on the isolation of several additional but unidentified pyrrolpyrazine alkaloids in Neotyphodium-infected Festuca and Poa species.

4.5. Organic Acids

Symbiotic plants of specific cultivars also contain significantly higher concentrations of γ-aminobutyric acid (Porter, 1994), which increases along with butyric acid following leaf harvest or death (C. W. Bacon, and G. B. Garner, unpublished data). Butyric acid and γ-aminobutyric acid (as the betaine) have been reported to be nematicidal agents produced by the neem tree, algae, and decomposing rye and timothy plants (Wu et al., 1997, 1998; Crouch et al., 1993). Moreover, the direct additions of these and other organic acids to soils were effective in controlling nematodes (Johnston et al., 1959; Patrick et al., 1958, 1965; Banage et al., 1965; Wilkinson and Mays, 1979; McBride et al., 2000). These soil organic acids were shown to be toxic to soil nematode levels of M. javanica, M. incognita, Tylenchorhynchus martini, Rhabditis terestris, and Dorylaimus sp, which strengthens their roles as biologically active components against nematodes in symbiotic tall fescue. We do not know the in planta distribution of these organic acids, nor do we know if there are any contributions from them to the soil with resulting nematicidal properties observed by others. The finding that they are present in higher levels in symbiotic tall fescue suggests at least an in-situ accumulation pattern reflective of some plant–endophyte interactions, in addition to accumulation in soil following leaf-litter decomposition. The occurrence of excessive amounts of organic acids, individually and collectively, has not been measured in fungal extract. Interestingly, the β-carboline alkaloids (harman and norharmane) have γ-aminobutyric acid-like activity, i.e., they alter γ-aminobutyric receptor functions, which if present in nematodes, would have a tremendous effect on nematode plant colonizing and soil migratory abilities.

4.6. Perloline

Perloline is an alkaloid found in several grass species in which it is found in very high concentrations and it is the predominant alkaloid in several cultivars of tall fescue, especially Kenwell and Kenhy (Asay et al., 1979). Its presence in both infected and uninfected tall fescue demonstrate that it is under genetic control and indicate that this substance is a plant product. High perloline concentration in plants is recessive to low perloline concentration. Further, the concentration of perloline varied (2000–3000 μg/g dried wt.), and depended on environmental soil factors, as well as growth stage of the plant (Cunningham and Clare, 1943; Bush et al., 1979). For example, the concentrations of this and other alkaloids are greatest in the stem, followed by the root, and then leaves, but during vegetative
regrowth the roots contained a significantly higher concentration of this alkaloid (Gentry et al., 1969). The concentration of perloline can be increased eightfold by nitrogen fertilization.

Perloline was initially associated with cattle toxicities, but a grass cultivar developed for very low perloline concentrations was exceedingly more toxic to cattle than grasses containing very high levels of perloline. It has not been established if perloline accumulation in tall fescue roots is endophyte-related, suggesting that there might be a quantitative response to infection and to any resulting nematode-deterring or toxic activity. There are no data indicating if in fact endophyte-infected grasses are higher than symbiotic plants. Perloline exhibits no mammalian toxicity, although it inhibited in-vitro ruminal cellulose digestion and cellulytic bacteria in cattle and lambs (Bush et al., 1972; Yates, 1983). Nevertheless, the high, and uniform distribution of perloline throughout the plant axis, and higher concentrations in roots during plant regrowth, makes it a prime candidate as a nematode toxin.

4.7. Lolitrems

Lolitrem B is a neurotoxic indole diterpenoid and is the cause of ryegrass staggers in sheep and cattle. There are several lolitrems as well as structurally related diterpenoids in plants. Paxilline, a precursor to lolitrem B, is usually found in higher concentrations than the lolitrems, and has been shown to be a feeding deterrent and is toxic to Argentine stem weevils. Lolitrem B is the most abundant of the lolitrems, and it is found in perennial ryegrasses and F. longifolia infected with N. lolli and E. fescuecae, respectively. The in planta concentrations are greater in the leaf sheath than in the blade. In addition to their tremorgenic effects, the indole diterpenoids exhibit differential toxicities to several mammalian species, and are either toxic or deterrents to insects (Prestidge and Ball, 1993). These substances also modulate calcium-activated potassium channel activity (Knaus et al., 1994), and they inhibit acyl-CoA cholesterol acyl transferase (Hung et al., 1995).

5. MECHANISMS OF ACTION

5.1. Fungal Toxins

Before any discussion on how symbiotic grasses may control plant parasitic nematodes, consideration must be given to the nature of the interaction of both the fungus–plant and nematode. The multifaceted expressions of fungal endophytes increase the phenotypic diversity of grasses within a population, contributing to the overall fitness of that population to survival under prevailing conditions at a location (Aracheveleta et al., 1992). Genetic expressions of the fungus interact with that of the host, which also diverge within the population, and the overall
expression is controlled by the host. Host control of toxic secondary metabolites is documented with the study of host control over the accumulation of ergot alkaloids (Agee and Hill, 1994; Hill et al., 1991; Hill and Brown, 2000; Roylance et al., 1994). Thus, the final expression of a toxin or other control measures for deterring nematode activity is an integrated and complex interaction between the host genome and the fungus genome. Variation of any type of fungal toxin within a population of grasses is expected.

The life cycles of the various types of nematodes are complex. Plant parasitic nematodes spend parts of their life cycle on or in the plant, and the other part in the soil. Control mechanisms used by symbiotic grasses representing one ecotype may be effective in the control of cyst and root-knot nematodes and other sedentary endoparasitic species, but might be totally ineffective against ectoparasitic species. Furthermore, not only is the species of nematode important in considering the broad range of control by symbiotic grasses, but also the specific stage or stages in the pest’s life cycle is important as a target for control. Therefore, the activity of an endophytic toxin on second-stage juveniles, males, or adult males and females must be tested. Also, the fecundity of a species and how many generations are produced during the season are important parameters for determining the efficacy of a control agent.

Since the symbiotic interactions of grasses are highly variable for each specific defensive toxin, it is up to the researcher to find that important combination that can be exploited for the desired control. The approach used to define nematode resistance and control should involve extensive surveys of clonal types conducted under field conditions where the desired nematode species exist. Thus, nematode resistance to specific species should be conducted in symbiotic grass fields in soils that have been shown to have that nematode. A clonal line within this location must be checked for its endophyte status, since other factors, biotic and abiotic, might also account for apparent nematode resistance, as demonstrated by the results of Bernard et al. (1998) and reflected in the report of Cook et al. (1999), which identified several ryegrass clones that were resistant to *M. naasi*, although the final analysis showed that all were endophyte-free.

### 5.2. Endophyte-Induced Resistance to Nematodes

It is envisioned that the defensive systems of plants include preformed physical and chemical barriers as well as inducible defenses that also can be either physical or chemical. An example of an inducible defense is the synthesis of specific antimicrobial compounds such as phytoalexins or phytoanticipins. However, in other instances plants react to pests by developing long-lasting, broad-spectrum systemic resistance to later attacks by pathogens. This type of resistance is referred to as systemic acquired resistance, and was discovered in tobacco by Ross (1961). This form of resistance is almost universally
characterized by the production of salicylic acid, which is used as an indicator. This increase in salicylic acid is also associated with the infection and time of infection as well as the increase in the synthesis of pathogenesis-related proteins, including β-glucanase, chitinase, other proteases, peroxidases, and lipoxygenases. In addition to salicylic acid, other chemicals are being sought that can induce systemic acquired resistance in the absence of phytotoxicity, which if discovered would be important for crop protection in general.

Systemic acquired resistance is an important facet of biocontrol for other endophytic microorganisms, especially endophytic bacteria (Hallmann et al., 1997b). The use of systemic acquired resistance for nematode control would depend on nematode resistance provided by the response, but demonstration that systemic acquired resistance occurs in symbiotic plants is incomplete (Roberts et al., 1992). However, the control of several nematode species by symbiotic tall fescue is highly suggestive of systemic acquired resistance (Roberts et al., 1992). The spectrum of pathogens being controlled by systemic acquired resistance includes viruses, bacteria, fungi, nematodes, and insects (van Loon et al., 1998), indicating how effective such a system is for complete disease control. The induction of this process in plants by an endophytic fungus should make the process more effective than chemical applications used for inducing other acquired resistance in other plants. Complete documentation that systemic acquired resistance occurs in symbiotic grasses and research as to the mechanism of pest and nematode resistance should involve examination of the secondary metabolites of the endophyte, such as peramine or ergot alkaloids, on induction of resistance. However, these chemicals might be related to resistance in plant fungal/bacterial disease defense, and not nematodes. The effects of endophyte status on fungal/bacterial disease are phenomena that have not established. Alternatively, the use of specific enzymes, and chemicals used as indicators for systemic induced resistance, might be tried. Roberts et al. (1992) reported an increase in chitinase activity if infected tall fescue, which is one enzyme used to document induced systemic resistance. The nematode *M. marylandi* was used to determine the increase in chitinase activity (Roberts et al., 1992), which if substantiated should play a major role in studying migratory endoparasitic nematodes, as well as sedentary endoparasitic and cyst nematodes.

Finally, there also may be an interaction of plant compounds with other endophyte-specific compounds that serve as effective nematode-deterring or toxic substances. For example, foliar chitinase is considered to play a role in nematode resistance (Roberts et al., 1992). Other examples include the work of Breen (1994). When *M. marylandi* was inoculated in soil, foliar chitinase activity was 10 times higher in the treatment groups than in controls. Further, in Kentucky 31 tall fescue clones, chitinase activity is high in resistant clones and low in susceptible ones. In this same experiment, Johnstone, a cultivar known for its lack of field persistence, showed low levels of chitinase and did not survive the...
nematode treatment, while symbiotic Kentucky 31 showed high levels of chitinase and survived the entire experiment. It is not known if the level of chitinase is simply correlated with nematode resistance or involved directly in a resistance mechanism.

6. SUMMARY

The discussion above indicates that as a result of interactions among the fungus, host, and nematode species, the mechanism of action may be difficult to predict. There is also the possibility that a combination of these with other unknown factors may form the basis for nematode toxicity and control. There may be more than one mechanism for the control of nematodes, and this would depend on the nature of selecting pressure to which a population of grasses has been subjected. Any of the fungal toxins or plant metabolites described above, alone and in combination, are candidates for control of nematods. No tests of these compounds on nematode activity have been made. There is also the possibility of an unidentified nematode-specific toxin. A valid approach designed to answer the general question of how an endophyte controls nematode has not been approached experimentally, and might prove difficult since we now know that there are also variations to each and every biotic and abiotic response within any symbiotic population of tall fescue, reflective of both a fungus genotype and a host genotype (Bacon, 1988; Agee and Hill, 1994; Hill et al., 1991; Hill and Brown, 2000; Roylance et al., 1994). The specific way by which the plants determine the level of endophyte expression has not been investigated, but it is known that the level of nitrogen administered to plants affects the in planta concentration of ergot alkaloids (Arachevaleta et al., 1992). Soil types are also important in determining the expression of any nematode toxin, since they determine the rate of growth and reproduction on plants. Nevertheless, with the technology available we can now dissect these complexities into their individual components, study each, culture and chemically test these with either in-vitro or in-vivo nematode bioassays, and reinsert the desired endophyte into grasses for the desired effect. Indeed, there may be endophyte–grass combinations that can produce a broad-spectrum toxicity to all types of nematodes, extending the use of endophytes as biological control agents. Symbiotic grasses have exploitive and biotechnological values far beyond the initial questions of their involvement in livestock toxicity syndromes. The question now develops: Do symbiotic grasses such as tall fescue have any economically important effects on nematodes, and if so, is it a form of resistance and therefore highly specific, or is it acutely toxic and very broad in its effect?
REFERENCES


