Preface

Since the initial report of specific DNA amplification using the polymerase chain reaction (PCR) by Kary Mullis and co-workers in 1985, the number of different applications of the technique has grown exponentially. The technique is now considered to be indispensible to molecular biology applications in every field of modern biology. The PCR technique is extremely sensitive and makes it possible to greatly amplify selected DNA sequences from very small quantities of template DNA. Its rapid development and diverse applications have allowed PCR to join other important molecular biology techniques, such as Southern blotting, gene cloning, pulsed field gel electrophoresis, etc., and has literally transformed the way that biologists think about approaching fundamental and applied biological problems. Indeed, PCR’s capacity to amplify specific segments of DNA represents a technology that has revolutionized molecular biology.

The purpose of this book is to highlight the many diverse applications that PCR has in basic and applied mycology. The editors have assembled an international group of world-renowned mycologists to illustrate the many application areas of PCR to their specialized fields of applied mycology. It is our hope that the comprehension of this material by the readers will enhance their understanding of the technology and help them to gain new appreciation for the many potential benefits of PCR application.

The text is composed of 15 chapters devoted to PCR principles and applications in a variety of diverse mycological areas. The opening chapter presents a brief overview, and the last chapter (Chapter 15) highlights potential future directions of PCR in mycology. Chapters 2 and 3 cover the many uses of PCR in fungal gene cloning and fungal gene expression. The material presented in Chapter 4 is important because it shows the utility of
PCR in explaining fungal speciation in a more definitive manner. Chapters 5–13 demonstrate the diverse application of PCR to studies of lichen mycobionts (Chapter 5), mycorrhizal fungi (Chapter 6), entomopathogenic fungi (Chapter 8), mycotoxin-producing fungi (Chapter 11) and its numerous applications in plant-fungal interactions (Chapter 13). Chapter 7 presents an interesting review of the use of PCR amplification in helping to determine fungal phylogeny more accurately. Chapters 9 and 10 highlight practical applications of PCR in fungal lignocellulose degradation and its use in the development of highly productive strains of industrially important fungi. Chapters 12 and 14 stress the significance of PCR in medical mycology and in providing a better understanding of the seed-borne disease state.

We are grateful to the many international authorities and specialists in mycology who have graciously consented to share their perspectives and expertise on the diverse applications of PCR amplification in their specialized mycological fields. We are also indebted to Tim Hardwick of CABI PUBLISHING for his continuing encouragement and guidance.

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Abbreviations

6-APA 6-aminopenicillanic acid
AFLP amplified fragment length polymorphism(s)
AG anastomosis group
AM arbuscular mycorrhizal
AP-PCR arbitrarily primed PCR
BAL bronchoalveolar lavage
BLO bacteria-like organism
bp base pairs
CBH cellobiohydrolases
CDH cellobiose dehydrogenase
cDNA complementary DNA
CTAB cetyl-trimethyl ammonium bromide
DAF DNA amplification fingerprinting
DD differential display
DDRT–PCR differential display reverse transcription PCR
DENS differential extension with nucleotide subsets
DGGE denaturing gradient gel electrophoresis
DMSO dimethylsulphoxide
dNTPs deoxyribonucleotide triphosphates
ds DNA double-stranded DNA
ECM ectomycorrhizal
EIPCR enzymatic inverse PCR
ELISA enzyme-linked immunosorbent assay
ERIC enterobacterial repetitive intergenic consensus
EST expressed sequence tag
GLOX glyoxal oxidase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>GM</td>
<td>galactomannan</td>
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<td>GRAS</td>
<td>generally recognized as safe</td>
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<td>GUS</td>
<td>β-glucuronidase</td>
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<td>HV</td>
<td>highly virulent</td>
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<td>IA</td>
<td>invasive aspergillosis</td>
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<td>IGS</td>
<td>intergenic spacer</td>
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<td>IPCR</td>
<td>inverse PCR</td>
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<tr>
<td>ISG</td>
<td>intraspecific group</td>
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<tr>
<td>ITS</td>
<td>internal transcribed spacer</td>
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<tr>
<td>LA</td>
<td>latex agglutination</td>
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<td>LA-PCR</td>
<td>long and accurate PCR</td>
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<td>LCR</td>
<td>ligase chain reaction</td>
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<td>LIC</td>
<td>ligase-independent cloning</td>
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<td>LIP</td>
<td>lignin peroxidase</td>
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<td>LM-PCR</td>
<td>ligation-mediated PCR</td>
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<td>LSU</td>
<td>large subunit</td>
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<td>MNP</td>
<td>manganese-dependent peroxidases</td>
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<td>mp</td>
<td>most parsimonious</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>NASBA</td>
<td>nucleic acid sequence-based amplification</td>
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<td>PAH</td>
<td>polyaromatic hydrocarbon</td>
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<td>PCP</td>
<td><em>Pneumocystis carinii</em> pneumonia</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDI</td>
<td>protein disulphide isomerase</td>
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<td>PFGE</td>
<td>pulsed-field gel electrophoresis</td>
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<td>POAc</td>
<td>phenoxyacetic acid</td>
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<td>PVA</td>
<td>penicillin V amidase</td>
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<td>QB</td>
<td>Q-beta replicase</td>
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<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
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<td>RAMS</td>
<td>random amplified microsatellites</td>
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<td>RAPD</td>
<td>random amplified polymorphic DNA</td>
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<td>RDA</td>
<td>representational difference analysis</td>
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<td>rDNA</td>
<td>ribosomal DNA</td>
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<tr>
<td>REP</td>
<td>repetitive extragenic palindromic</td>
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<td>REP-PCR</td>
<td>repetitive-element based PCR</td>
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<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
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<tr>
<td>RPCR</td>
<td>recombination PCR</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription PCR</td>
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<td>SCAR</td>
<td>sequence characterized amplified regions</td>
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<td>SDA</td>
<td>strand displacement amplification</td>
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<td>SEAD</td>
<td>selective enrichment of amplified DNA</td>
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<td>SQPCR</td>
<td>semi-quantitative PCR</td>
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<td>SSR</td>
<td>simple sequence repeat</td>
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<tr>
<td>SSU</td>
<td>small subunit</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>STR</td>
<td>short tandem repeat</td>
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<tr>
<td>STS</td>
<td>sequence-tagged sites</td>
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<tr>
<td>TB</td>
<td>tuberculosis</td>
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<tr>
<td>TdT</td>
<td>terminal deoxynucleotide transferase</td>
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<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
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<tr>
<td>$T_m$</td>
<td>melting temperature</td>
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<tr>
<td>TNV</td>
<td>tobacco necrosis virus</td>
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<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
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<tr>
<td>TS</td>
<td>thymidylate synthase</td>
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<tr>
<td>VNTR</td>
<td>variable non-translated repeats</td>
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<tr>
<td>WV</td>
<td>weakly virulent</td>
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1.1 Overview of PCR Methods

The polymerase chain reaction (PCR) is a powerful method with widespread applications in molecular biology. This enzymatic reaction allows in vitro amplification of specific DNA fragments from complex DNA samples and can generate microgram quantities of target DNA. Any nucleic acid sequence can be cloned, analysed or modified, and even rare sequences can be detected by PCR amplification. Since its development in 1985, the specificity, sensitivity and speed of this technology have led to the development of many methods for a wide range of biological research areas and for all classes of organisms. Extensive applications have been found for PCR in many fields of mycology, including fungal genetics and systematics, ecology and soil microbiology, plant pathology, medical mycology, fungal biotechnology and many others. Moreover, it is certain that fungal studies will continue to progress with PCR, as numerous improvements, modifications and new applications are regularly reported.

1.2 PCR: the Standard Method

1.2.1 Principles of PCR

The polymerase chain reaction allows the exponential amplification of specific DNA fragments by in vitro DNA synthesis (Saiki et al., 1985; Mullis et al., 1986; Mullis and Faloona, 1987). The standard method requires a DNA template containing the region to be amplified and two oligonucleotide
primers flanking this target region. The amplification is based on the use of a thermostable DNA polymerase isolated from *Thermus aquaticus*, called Taq polymerase (Saiki et al., 1988). All PCR reaction components are mixed and the procedure consists of a succession of three steps which are determined by temperature conditions: template denaturation, primer annealing and extension (Fig. 1.1). In the first step, the incubation of the reaction mixture at a high temperature (90–95°C) allows the denaturation of the double-stranded DNA template. By cooling the mixture to an annealing temperature which is typically around 55°C, the target-specific oligonucleotide primers anneal to the 5' end of the two single-stranded templates. For the extension step, the temperature is raised to 72°C and the primer–target hybridizations serve as initiation points for the synthesis of new DNA strands. The time incubation for each step is usually 1–2 min. This sequence of three steps corresponds to one cycle of PCR. In the second cycle, the newly synthesized DNA strands are separated from the original strands by denaturation and each strand serves again as template in the annealing and extension steps. Theoretically, \( n \) cycles of PCR allow a \( 2^n \)-fold amplification of the target DNA sequence. Typically, PCR is carried out for 30–40 cycles. As all components including the thermostable DNA polymerase are present in the PCR mixture from the beginning of the reaction, the amplification procedure can be automated and performed in a thermocycler with programmed heating and cooling.

### 1.2.2 PCR reaction components and conditions

The template DNA, oligonucleotide primers, DNA polymerase and deoxyribonucleotide triphosphates (dNTPs) are mixed in an appropriate buffer containing magnesium ions (as MgCl\(_2\)). The volume of the reaction mixture generally ranges from 25 to 100 µl. Standard conditions for the concentration of the different components are given in Table 1.1. These allow amplification of most target sequences but can be optimized for each new PCR application (Innis and Gelfand, 1990). As an example, the starting concentration of MgCl\(_2\) is generally 1.5 mM for amplification with Taq DNA polymerase. If satisfactory PCR results are not obtained, this concentration can be optimized: a higher MgCl\(_2\) concentration will increase the yield of amplified products but decreases the specificity; a lower MgCl\(_2\) concentration increases the specificity but decreases the yield. Similarly, the temperature and time conditions for each step of the reaction, especially the annealing step, should be optimized for each target sequence and primer pair. The annealing temperature is generally optimized empirically, by increasing the temperature until the best result is obtained in terms of amplification yield and specificity.

Classical PCR amplifications require minimal sequence information from which appropriate primers are selected. Primers are generally 18–28 nucleotides in length and are defined according to the DNA sequences flanking the
Fig. 1.1. Principle of PCR amplification.
region to be amplified. Primers are written in the order 5'→3' (Fig. 1.2). Two parameters are important for the primer design: the efficiency and the specificity of amplification. For instance, an important rule is that no complementarity between 3' ends of the primers should exist, to avoid formation of primer-dimers that would decrease the yield of the amplified product. General concepts for primer design have been described (Dieffenbach et al., 1993; He et al., 1994; Rychlik, 1995). The specificity of primer matching can also be improved by adding particular compounds in the reaction mixture.

**Table 1.1.** Standard conditions for PCR amplification: concentration of the different PCR components.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Template DNA</td>
<td>10–100 ng</td>
</tr>
<tr>
<td>Amplification buffer</td>
<td>1/10 of final volume (buffer is supplied 10× concentrated with the Taq DNA polymerase)</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.5–5 mM (typically 1.5 mM)</td>
</tr>
<tr>
<td>dNTPs</td>
<td>20–200 µM each of dATP, dCTP, dGTP and dTTP</td>
</tr>
<tr>
<td>Primer 1</td>
<td>0.1–0.5 µM</td>
</tr>
<tr>
<td>Primer 2</td>
<td>0.1–0.5 µM</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.5–2.5 units</td>
</tr>
<tr>
<td>Sterile distilled water</td>
<td>To final volume</td>
</tr>
<tr>
<td>Final volume</td>
<td>25–100 µl</td>
</tr>
</tbody>
</table>

**Fig. 1.2.** Schematic representation of a target DNA region to be amplified by PCR and the sequence of the corresponding oligonucleotide primers.
Filichkin and Gelvin, 1992) or by using particular experimental conditions such as ‘touchdown’ PCR (Don et al., 1991) or ‘hot start’ PCR (Chou et al., 1992). In a touchdown PCR, the first cycle is carried out with an annealing temperature which is higher than the expected annealing temperature (i.e. 10°C above). This temperature is then decreased by 1°C every PCR cycle until a ‘touchdown’ at the correct annealing temperature is reached: the remaining cycles are performed at this temperature. In a ‘hot start’ PCR, at least one of the essential components of the PCR reaction is physically separated from the amplification mixture by wax during the initial heat denaturation step. The first heating step melts the wax and allows complete mixing of all the components. This ‘hot start’ protocol prevents the non-specific binding of primers during the initial heat denaturation of the template.

The standard PCR uses Taq DNA polymerase but other thermostable DNA polymerases are available for PCR amplifications. Recombinant DNA polymerases have also been developed recently. The choice of DNA polymerase depends on the PCR application and the properties of the various enzymes (Arnheim and Erlich, 1992). As an example, the native Taq DNA polymerase lacks a 3’ → 5’ exonuclease (proofreading) activity and thus produces single base substitution errors at a relatively high rate (Tindall and Kunkel, 1988). By using other thermostable DNA polymerases which have this proofreading function, the fidelity of the DNA amplification can be increased (Cha and Thilly, 1993). Moreover, PCR is classically used to amplify target DNA sequences less than 4000 bp in length, but PCR protocols have recently been developed to amplify longer DNA fragments (Kainz et al., 1992; Cheng et al., 1994).

1.2.3 Practical considerations in PCR experiments

PCR reactions are classically performed in a final volume of 25–100 µl, in which small volumes of each reagent have to be added. These multiple pipetting steps increase the possibility of errors and inaccuracy. As multiple samples are generally amplified using the same conditions, it is recommended that a mix containing all reagents for all samples except the template be prepared, and that this mix is then distributed into individual tubes before finally adding the template DNA.

Because of the ability of the PCR to amplify DNA sequences present at a small number of copies, contamination of the template DNA mixture with a foreign source of DNA can result in the amplification of both the target and the contaminant, especially when non-specific primers are used. The presence of PCR products and primers from previous amplifications can also result in carry-over contaminations. In order to detect false-positive amplifications due to contamination, each PCR experiment must include a negative control which contains the same mixture of reagents as the other samples but without template DNA, so that no amplification should occur in the negative control. PCR experiments should also include a positive control (with a
reference template DNA known to amplify) as false-negative amplifications can also occur because of problems with the thermocycler, the reagents or inhibition of the DNA polymerase.

Some precautionary measures should be taken to minimize the risk of contamination in PCR. A first recommendation is to designate a specific area of the laboratory and a specific set of pipettors for the preparation of PCR reactions and always to use sterile tubes and pipette tips and wear disposable gloves. These precautions can be improved by carrying out PCR reactions in a hood equipped with ultraviolet light and to use UV irradiation of the hood before handling PCR components. Furthermore, the different reagents used for PCR can also become contaminated and to avoid this small aliquots of stock solutions of primers, dNTPs and buffer, and aliquots of sterile water should be prepared. This will avoid multiple pipetting from the same stock tube, and allows the use of new aliquots if a problem should arise. Other specific procedures have also been proposed to minimize contamination of reactions, such as the use of dUTP instead of dTTP and uracil DNA glycosylase (Longo et al., 1990) or gamma-irradiation (Deragon et al., 1990).

1.2.4 Analysis of PCR products

In many applications, PCR products need only to be visualized. They are separated electrophoretically according to their size on agarose gels, or polyacrylamide gels if higher resolution is required, and visualized by ethidium bromide or silver staining. More sensitive detection of the PCR products can be achieved by hybridization with a specific probe or by labelling the product either after amplification or directly during PCR using labelled dNTPs or primers (Lo et al., 1990; Höltke et al., 1995; Inazuka et al., 1996). Other approaches such as the use of capillary electrophoresis allow product analysis to be automated (Martin et al., 1993). Capillary electrophoresis provides a size-selective separation of DNA fragments and results can be displayed as an electrophoregram. The addition of a standard size ladder of known concentration to the DNA sample to be analysed allows the determination of both the size and the quantity of the PCR products.

Once identified, target DNA generated by PCR amplification can be further characterized by various genetic analyses. Several strategies have been developed for the direct sequencing of PCR-amplified DNA fragments (Bevan et al., 1992; Rao, 1994). Instead of sequencing, denaturing gradient gel electrophoresis (DGGE) can be used for direct analysis of amplified DNA. This technique is especially useful for the detection of mutations in a target sequence since it permits the separation of DNA products of the same length but with different nucleotide sequences and can be used to distinguish single-base substitutions (Fisher and Lerman, 1983; Sheffield et al., 1989). Similarly, single-strand conformation polymorphism (SSCP) analysis (Orita et al., 1989; Fujita and Silver, 1994) is a powerful electrophoretic method for detecting sequence variations and point mutations in amplified products. In
contrast to DGGE, SSCP analysis is performed in a polyacrylamide gel under non-denaturing conditions but DNA samples are denatured prior to electrophoresis. Variation in PCR products can be shown by restriction endonuclease digestion. The PCR products obtained from different strains can be digested with several enzymes and their resulting digests separated by electrophoresis. If sequence differences are located within restriction sites for particular enzymes, the digestion of the PCR products with those enzymes will lead to different electrophoretic patterns. This strategy of PCR–restriction fragment length polymorphism (RFLP) is suitable for screening large samples and is commonly used in taxonomic and ecological studies.

### 1.3 PCR-derived Methods

Since the first description of the PCR, several modifications of the original procedure have been developed. The sensitivity and specificity of the amplification have been improved by simple modifications of the standard procedure such as ‘nested PCR’, in which the PCR product is subjected to a second round of amplification with a second pair of oligonucleotide primers located internally to the first pair (Dieffenbach et al., 1993). ‘Quantitative PCR’ methods allow the estimation of the number of target DNA sequences, generally by competitive PCR with an internal standard (Cross, 1995). Further developments which are more than simple variations of the original procedure include modifications of the type of template (RNA instead of DNA) or the type of primers (randomly chosen instead of target-specific primers), and these have led to new methods which have increased considerably the range of applications of PCR technology. Finally, the recent development of in situ PCR methods is promising for both diagnosis and genetic analysis.

#### 1.3.1 Reverse transcription–PCR (RT–PCR)

PCR has been adapted to amplify RNA sequences such as mRNA and viral RNA by RT–PCR. Originally, the procedure was based on the reverse transcription (RT) of the RNA into cDNA by a reverse transcriptase prior to amplification by Taq polymerase and standard reaction conditions were described by Kawasaki (1990). Direct RNA amplification with a single enzyme is also possible by using a particular polymerase (Tth) from Thermus thermophilus (Myers and Gelfand, 1991). This polymerase has a reverse transcriptase activity in the presence of manganese ions and a DNA polymerase activity in the presence of magnesium ions.

RT–PCR is a highly sensitive method that allows the detection and the analysis of very small amounts of specific RNAs such as previously undetectable rare transcripts (Ohan and Heikkila, 1993). Moreover, quantitative RT–PCR approaches have been developed for measuring gene expression (Gilliland et al., 1990; Riedy et al., 1995; Gibson et al., 1996).
1.3.2 Random PCR and other PCR fingerprinting methods

In contrast to ‘classical’ PCR, random PCR approaches do not require any nucleotide sequence information for primer design and allow amplification of DNA fragments which are of undefined length and sequence. Among these approaches, random amplified polymorphic DNA (RAPD) analysis (Williams et al., 1990) was first developed to detect polymorphisms between organisms despite the absence of sequence information, and to produce genetic markers and to construct genetic maps. This method has also been called arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990) and DNA amplification fingerprinting (DAF) (Caetano-Anollés et al., 1991). The method is based on the PCR amplification of genomic DNA with a single short primer with an arbitrary nucleotide sequence. The PCR is carried out with a low annealing temperature and generates several PCR products which produce a band pattern after electrophoretic separation.

Because RAPD assays are generally performed under low stringency conditions (i.e., low annealing temperatures) with non-specific primers, they are more sensitive than conventional PCR assays to reaction and thermocycle conditions and thus the concentration of all components in the reaction mixture must be accurately standardized. Moreover, the quality of template DNA and the brand of Taq DNA polymerase and thermocycler used are also factors that can affect the reproducibility of RAPD results (MacPherson et al., 1993; Tommerup et al., 1995).

Another random PCR approach that has recently been developed for DNA fingerprinting and genetic mapping is the amplified fragment length polymorphism (AFLP) technique (Vos et al., 1995). This procedure allows high stringency PCR amplification of DNA fragments randomly chosen from restriction fragments. In this technology, genomic DNA is first digested with a restriction endonuclease and oligonucleotide adaptors are ligated to the ends of the restriction fragments. Then, a PCR amplification is performed using primers which include the adaptor sequence, the part of the restriction site sequence remaining on the fragment, and between one and five additional nucleotides which are randomly chosen. This step allows selective amplification of the restriction fragments in which the nucleotides flanking the restriction site match the additional nucleotides of the primers. The amplified fragments are analysed by denaturing polyacrylamide gel electrophoresis to generate the fingerprint. The AFLP technique has two main advantages: it is similar to RAPD in that it analyses the whole genome but it differs from RAPD in that it uses stringent PCR conditions and produces results that are very reproducible. AFLP also has high resolution and the complexity of the AFLP fingerprint can be planned since the number of resulting fragments depends on the choice of restriction enzyme and the number of additional selective nucleotides in the primers.

Other PCR-based methods are intermediate between random PCR approaches and target-directed amplifications. Indeed, in RAPD analysis,
arbitrary primers which are used generally match repeated elements in the DNA since a multiple banding pattern is expected in the resulting PCR fingerprint. PCR fingerprinting methods can also be based on the amplification with primers defined according to known repeated elements (Meyer et al., 1993a; van Belkum, 1994) and this strategy is also termed interrepeat PCR. Primers can be directed against microsatellites or minisatellites, which are tandemly repeated motifs of 2–10 bp, or 15–30 bp, respectively (Meyer et al., 1993b), or against other eukaryotic or prokaryotic repeated motifs (van Belkum et al., 1993). For instance, PCR primers corresponding to enterobacterial repetitive intergenic consensus (ERIC) sequences and repetitive extragenic palindromic (REP) elements (Versalovic et al., 1991) can be used to fingerprint the genome of microorganisms. PCR fingerprinting methods are now widely used to characterize genetic variations within almost any type of organism including fungi.

1.3.3 In situ PCR

Biological diagnostics have been greatly advanced by both PCR and in situ hybridization. While the main advantage of PCR is its high sensitivity, in situ hybridization allows the detection and cytological localization of nucleic acid sequences in whole cells. In situ PCR, resulting from the combination of PCR and in situ hybridization, is a powerful method for the identification and localization of rare DNA sequences or RNA sequences (after RT–PCR) in whole cells or tissue samples. In situ PCR methods include the following steps: sample fixation, sample permeabilization to allow penetration of PCR reagents, in situ amplification, and visualization of the PCR products by in situ hybridization. Alternatively, PCR products can be visualized directly when labelled nucleotides are incorporated during the amplification step; however, the reaction is generally less reliable and less specific by direct in situ PCR than indirect in situ PCR with hybridization (Long et al., 1993). Protocols for PCR – in situ hybridization and RT – in situ PCR have recently been reviewed by Nuovo (1994). In situ PCR has numerous applications in the identification and localization of specific DNA and RNA sequences in intact cells, disease diagnosis and quantification, and in the analysis of infection process and gene expression. Until now, most in situ PCR assays developed have been concerned with the detection of viral sequences in infected cells.

1.4 PCR Alternatives

In the last few years, many other methods for nucleic acid amplification have been developed, such as the ligase chain reaction (LCR), nucleic acid sequence-based amplification (NASBA), Q-beta replicase (Qβ) amplification, or strand displacement amplification (SDA). Further details of these
procedures can be found in Winn-Deen (1996). These can be used instead of or in combination with PCR, and were generally developed for diagnostic purposes. For instance, LCR (Barany, 1991; Wiedmann et al., 1994) is a DNA amplification system which involves the use of four oligonucleotide primers and a thermostable DNA ligase. This method, often combined with PCR, is a powerful method for the detection of genetic diseases resulting from mutations since it allows the discrimination of DNA sequences differing in only a single base pair. The NASBA system (Kievits et al., 1991), was developed for the detection and amplification of RNA, and requires two primers and three different enzymes (reverse transcriptase, T7 RNA polymerase and RNase H). Unlike PCR, no thermocycler is needed since the NASBA reaction is performed at a constant temperature. Thus, NASBA is particularly suitable for diagnostic procedures which involve the analysis of large numbers of samples in a short time; it has already been applied to the detection of various viral infections.

1.5 Applications of PCR in Mycology

1.5.1 Starting PCR with fungi

The first stage in PCR is the preparation of the template DNA. Several protocols for the extraction of nucleic acids from fungi are available. Some of these allow the isolation of microgram quantities of pure genomic DNA (Garber and Yoder, 1983; Raeder and Broda, 1985; Rogers and Bendich, 1988) which is suitable for restriction enzyme analysis and other molecular applications. However, because only small quantities of starting DNA are required for PCR amplification, simplified and rapid procedures can generally be used (Lee and Taylor, 1990; Cenis, 1992; Steiner et al., 1995). DNA can also be extracted from complex environmental samples such as soils and plants (Porteous et al., 1994; Volossiouk et al., 1995; Zhou et al., 1996) or clinical samples (Makimura et al., 1994; De Barbeyrac et al., 1996), allowing direct amplification of fungal DNA from the mixed DNA extracts by the use of specific primers. These techniques are particularly useful for ecological studies and for the detection of fungi in various samples without preliminary isolation.

1.5.2 Taxonomy and characterization of fungi with PCR

One of the first applications of PCR in mycology was described in 1990 by White et al. and concerned the amplification and direct sequencing of ribosomal DNA (rDNA) to establish the taxonomic and phylogenetic relationships of fungi. rDNA sequences are often used for taxonomic and phylogenetic studies because they are found universally in living cells in which they have an important function; thus, their evolution might reflect the evolution of the whole genome. These sequences also contain both
variable and conserved regions, allowing the comparison and discrimination of organisms at different taxonomic levels. The nuclear rDNA in fungi is organized as an rDNA unit which is tandemly repeated. One unit includes three rRNA genes: the small nuclear (18S-like) rRNA, the 5.8S rRNA, and the large (28S-like) rRNA genes. In one unit, the genes are separated by two internal transcribed spacers (ITS1 and ITS2) and two rDNA units are separated by the intergenic spacer (IGS). The last rRNA gene (5S) may or may not be within the repeated unit, depending on the fungal taxa. The 18S rDNA evolves relatively slowly and is useful for comparing distantly related organisms whereas the non-coding regions (ITS and IGS) evolve faster and are useful for comparing fungal species within a genus or strains within a species. Some regions of the 28S rDNA are also variable between species.

The development of PCR and the design of primers for the amplification of the various rDNA regions has considerably facilitated taxonomic studies of fungi (White et al., 1990). These primers were designed from conserved regions, allowing the amplification of the fragment they flank in most fungi. The ITS primers designed by White et al. (1990) have enabled the determination of many ITS sequences from different fungi and these have been used to investigate taxonomic and phylogenetic relationships between species within different genera, such as Colletotrichum (Sreenivasaprasad et al., 1996a), Phytophthora (Lee and Taylor, 1992) and Penicillium (Lobuglio et al., 1993). ITS sequences are generally constant, or show little variation within species but vary between species in a genus, and so these sequences have been widely used to develop rapid procedures for the identification of fungal species by PCR–RFLP analysis (Vilgalys and Hester, 1990; Chen, 1992; Edel et al., 1997), and to design species-specific primers (Moukhamedov et al., 1994; Beck and Ligon, 1995; Sreenivasaprasad et al., 1996b). At the intraspecific level, differentiation of closely related fungal strains can be achieved by comparing more variable DNA regions such as the ribosomal IGS sequences, for which PCR primers have also been designed (Anderson and Stasovski, 1992; Henrion et al., 1992; Edel et al., 1995). Mitochondrial rDNA can also be easily analysed among fungi after amplification with consensus primers (White et al., 1990).

Random PCR approaches are being increasingly used to generate molecular markers which are useful for taxonomy and for characterizing fungal populations. The main advantage of these approaches is that previous knowledge of DNA sequences is not required, so that any random primer can be tested to amplify any fungal DNA. RAPD primers are chosen empirically and tested experimentally to find RAPD banding patterns which are polymorphic between the taxa studied. The RAPD method has been successfully used to differentiate and identify fungi at the intraspecific level (Guthrie et al., 1992; Assigbetse et al., 1994; Nicholson and Rezanoor, 1994) and the interspecific level (Lehmann et al., 1992). Similarly, PCR fingerprinting with primers that detect hypervariable and repeated sequences has been used to clarify the taxonomic relationships among both fungal strains and
species (Meyer et al., 1993b; van Belkum et al., 1993). As both RAPD and interrepeat PCR amplify DNA from non-specific primers, they need a pure DNA template and cannot be used to detect fungi in mixed samples. More recently, AFLP fingerprinting has been developed to evaluate polymorphisms among various organisms, and this has already been applied to the detection of inter- and intraspecific genetic variation in fungi (Majer et al., 1996; Mueller et al., 1996). AFLP has several advantages over RAPD in terms of reproducibility and the level of resolution per reaction, and the method has great potential for revealing variations among many fungi, especially at the intraspecific level.

1.5.3 PCR in the detection of fungi

Because of its specificity and sensitivity, PCR is an attractive method for the detection of fungi. There are already many examples of PCR-based assays developed for the detection of fungi in both medical and plant pathology. PCR can be used to detect groups of strains, pathotypes, species or higher taxa, provided that specific oligonucleotide primers for these taxa are available. Thus, the development of PCR-based detection procedures requires knowledge of sequences of at least a part of the target DNA region in order to design specific primers. The principle of these methods is the alignment of the sequences from target and non-target organisms and the selection of primers with mismatches to the non-target organisms but sufficient homology for efficient priming and amplification of all target organisms (Dieffenbach et al., 1993).

DNA sequences which are polymorphic between fungal species, such as ITSs, are good candidates for the detection of a species to the exclusion of all other species. For example, differences in ITS sequences have been used to develop PCR-based assays for the detection of many phytopathogenic fungal species in host plants without previous isolation of the fungi (Moukhamedov et al., 1994; Beck and Ligon, 1995; Goodwin et al., 1995; Sreenivasaprasad et al., 1996b). Other sequences of rDNA have been used to design specific primers, such as 18S rDNA (Di Bonito et al., 1995), 28S rDNA (Fell, 1995) and mitochondrial rDNA (Li et al., 1994). rDNA sequences have also been used to develop PCR assays for the detection of fungal species in clinical samples (Spreadbury et al., 1993; Holmes et al., 1994). As rDNA sequences are present in high copy number in the fungal genome, their use generally increases the sensitivity of a detection assay. Sequences unique to the target organisms can be found by other approaches. Specific primers can be designed from cloned genomic DNA fragments (Ersek et al., 1994; Doss and Welty, 1995) or from PCR-amplified specific fragments. Indeed, taxon-specific markers generated by RAPD or other PCR-fingerprinting methods can be cloned and sequenced, and these sequence-characterized amplified regions (SCARs) used to design specific primers for detection assays. An example of this is the detection of Fusarium species with primers designed...
from species-specific SCARs derived from RAPDs (Parry and Nicholson, 1996; Schilling et al., 1996). Finally, taxon-specific fragments generated by PCR fingerprinting or PCR products with specific sequences can be used as specific probes in diagnostic assays based on dot-blot hybridizations (Klassen et al., 1996).

PCR amplification methods with specific fungal primers are powerful tools not only in diagnostics but also in ecological studies for monitoring fungi in natural environments, such as water, soil, plant or clinical samples. Furthermore, the development of specific primers has greatly facilitated studies on obligate parasites and symbionts. For example, specific amplification of mycorrhizal fungal DNA can be performed from colonized plant roots (Di Bonito et al., 1995).

1.5.4 Genetic analysis and gene manipulations with PCR

The development of PCR methods has greatly facilitated genetic studies in all organisms. Many PCR-based methods are suitable for the analysis of genetic variants and to identify genetic markers. Sequence variation can be identified by analysis of a PCR product using RFLP, DGGE or SSCP, or direct sequencing and random PCR approaches. The detection of gene mutations such as deletions, insertions and even single-base substitutions can be achieved with the development of highly discriminatory methods for analysing and comparing PCR products. Polymorphisms detected by PCR and especially by RAPD are also useful for the construction of genetic maps and for genetic linkage analysis (Erlich and Arnheim, 1992; Tingey and del Tufo, 1993).

Cloning procedures, and thus gene isolation and manipulations, have also made use of the ability of PCR to produce large amounts of specific DNA fragments from a complex DNA mixture. Cloning of PCR products can be performed by using PCR primers that include restriction sites, which simplify the subsequent insertion and ligation of the cloned DNA fragment into the vector. Efficient protocols for cloning and analysis of blunt-ended PCR products have also been described (Costa and Weiner, 1994). Cloning strategies for genes encoding known proteins can be developed despite the absence of nucleotide sequence information normally required to design oligonucleotide primers, by using a mixture of degenerate primers consistent with the amino acid sequence (Compton, 1990). Degenerate primers may also be designed on the basis of nucleotide sequence alignment. Furthermore, inverse PCR approaches allow the isolation of DNA fragments which are adjacent to a known sequence (Silver, 1991). PCR strategies also allow in vitro gene construction and have greatly simplified site-directed mutagenesis (Weiner and Costa, 1994). For instance, PCR can be performed with oligonucleotide primers that allow both amplification and introduction of a mutation in the target sequence. This mutation may be an addition, a deletion or a substitution of nucleotides. The method is powerful enough for the study of relationships between the structure of a gene and its function.
Analysis of gene expression, and detection and quantification of RNA transcripts can be accomplished by PCR. More recently, differential display (DD) or DDRT–PCR (DD reverse transcription PCR) (Liang and Pardee, 1992) has been developed for genetic analyses. This technique allows the identification of differentially expressed mRNAs and the cloning and characterization of their genes, thus permitting the analysis of alteration of gene expression and the identification of genes that play important roles in biological and pathological processes (Liang et al., 1993; Goormachtig et al., 1995; Benito et al., 1996).

1.6 Conclusions

PCR procedures, from the standard method to its modifications and improvements, have greatly simplified DNA technologies. PCR-based methods offer so many possibilities that they are now used in almost all areas of life sciences. In mycology, many applications have already been described in taxonomy, phylogeny, population studies and diagnostics, and some examples have been reported in this chapter. However, PCR applications are being developed in many other fungal studies, including genetic analyses, biotechnology and the analysis of fungal–host interactions in both medical and plant pathology. The following chapters provide current reviews of many research applications of PCR in all these areas of mycology.

References


fingerprinting for the detection of genetic variation in fungi. Mycological Research 100, 1107–1111.


2.1 Introduction

Since its invention, the polymerase chain reaction (PCR) has revolutionized the methodology for cloning genes. To clone a gene of interest, only sequence information of two distinct regions of the gene (or the protein) is needed to enable the design of primers for the amplification of the intervening sequence. Following amplification of a putative gene fragment, the respective PCR product can be used as a homologous probe for cloning the complete gene from a library. PCR has therefore rapidly replaced the previously used screening of libraries with oligonucleotide probes. Due to the fast progress in PCR technologies and applications, further developments today enable cloning of a complete gene including 5′- and 3′-untranslated regions by PCR alone. This is particularly important in organisms where the preparation of genomic DNA in quantities and qualities sufficient for the construction of a genomic library is difficult. In this chapter we will attempt to review the current state of gene cloning by PCR.

2.2 Tools Required

2.2.1 Available DNA polymerases

Several commercial suppliers offer a variety of thermostable DNA polymerases which differ in their proofreading activity, thermostability and terminal deoxynucleotide transferase (TdT) activity (i.e., DNA polymerases which add an additional non-templated nucleotide to the 3′-end of a DNA
Table 2.1. Features of thermostable DNA polymerases.\(^{a,b}\)

<table>
<thead>
<tr>
<th>Feature</th>
<th>Taq</th>
<th>Stoffel</th>
<th>rTh</th>
<th>Pfu</th>
<th>Vent</th>
<th>Pwo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal extension temperature (°C)</td>
<td>75—80</td>
<td>75—80</td>
<td>75—80</td>
<td>72—78</td>
<td>76</td>
<td>72</td>
</tr>
<tr>
<td>Extension rate at 72°C (kb min(^{-1}))</td>
<td>2—4</td>
<td>2—4</td>
<td>2—4</td>
<td>0.5—1</td>
<td>1</td>
<td>1—2</td>
</tr>
<tr>
<td>Reverse transcriptase activity</td>
<td>Minimal</td>
<td>Minimal</td>
<td>Mn(^{2+}) dependent</td>
<td>Minimal</td>
<td>Minimal</td>
<td>Minimal</td>
</tr>
<tr>
<td>Half-life (min) at 100°C</td>
<td>&lt; 5</td>
<td></td>
<td></td>
<td>100</td>
<td></td>
<td>&gt; 120</td>
</tr>
<tr>
<td>Half-life (min) at 97.5°C</td>
<td>10</td>
<td>20</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Half-life (min) at 95°C</td>
<td>40</td>
<td>80</td>
<td>20</td>
<td>d</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>Mg(^{2+})-ion PCR optimum (mM)</td>
<td>1—4</td>
<td>2—10</td>
<td>1.5—2.5</td>
<td>1.5—2.5</td>
<td>1—6</td>
<td>1.5—4</td>
</tr>
<tr>
<td>pH optimum for PCR (25°C)</td>
<td>9.0</td>
<td>9.0</td>
<td>9.0</td>
<td>&gt; 8.0</td>
<td>8.8</td>
<td>8.8</td>
</tr>
<tr>
<td>dNTP PCR optimum (µM each)</td>
<td>40—200</td>
<td>40—200</td>
<td>40—200</td>
<td>100—250</td>
<td>200—400</td>
<td>200</td>
</tr>
<tr>
<td>KCl PCR optimum (mM)</td>
<td>50</td>
<td>10</td>
<td>75—100</td>
<td>10</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Primer PCR optimum (µM)</td>
<td>0.1—1</td>
<td>0.1—1</td>
<td>0.1—1</td>
<td>0.1—0.5</td>
<td>0.4</td>
<td>0.3—0.6</td>
</tr>
<tr>
<td>Longest PCR product (kb)</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
<td>25</td>
<td>15</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>dI-containing primers accepted</td>
<td>+</td>
<td></td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5′—3′ exonuclease activity</td>
<td>+</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3′—5′ exonuclease activity</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Error rate (mutation frequency per base pair per cycle (\times 10^{-6}))</td>
<td>8.0</td>
<td>1.3</td>
<td>2.7</td>
<td>e</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expandase activity</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Substrate analogues:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dUTP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>deaza-dGTP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>biotin-11-dUTP</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>digoxigenin-dUTP</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fluorescein-dUTP</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ddNTP</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bromo-dUTP</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-thionucleotides</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Continued on facing page
This enables the selection of the optimal polymerase for individual applications (Table 2.1). Polymerases with proofreading activity such as Pfu remove 3'-misincorporated nucleotides and so increase the accuracy of the reaction. Such polymerases may also increase the yield of amplicons as misincorporated nucleotides can inhibit the activity of the DNA polymerase (Barnes, 1994a). Conversely, proofreading activity can degrade primers (Lundberg et al., 1991; Flaman et al., 1994).

### 2.2.2 Source of DNA

An extensive search of literature from the past 5 years shows that most instances of the use of PCR in the cloning of fungal genes have used genomic DNA as a template. Since neither high quantities nor high quality of DNA are needed, a number of simple and rapid protocols can be used for DNA extraction. Virtually every type of fungal material can be used as a source of DNA, and procedures have been adopted for mycelia harvested from liquid cultures (Gruber et al., 1990), solid media (Lecellier and Silar, 1994), fruiting bodies, herbarium materials (Bruns et al., 1990) or from archaeological specimens (Rollo et al., 1995).

### 2.2.3 Primer design

Design of primers is one of the key steps in the successful cloning of a gene. However, no absolute rules can be given that will guarantee the amplification of the desired fragment in sufficient quantities. The following features may be taken as guidelines for the design of primer pairs: (i) the length of primers should be between 20 and 30 bases; (ii) the GC content should be around 50%, and G and C nucleotides should preferably be randomly distributed within the primer. Over-long GC-stretches should be particularly avoided at
the 3′-end of the primer, as this can cause efficient but unspecific priming at any GC-rich region in the genome (Innis and Gelfand, 1990); (iii) both primers should have a similar GC content to minimize differences in annealing at a given temperature; (iv) stretches of poly(dR), poly(dY) and palindromic sequences should be avoided; (v) the sequences of the two primers must not be complementary; (vi) the concentration of both primers in the reaction should be between 0.1 and 0.5 µM. Differences in the concentration of the two primers should be avoided since this would favour the amplification of a linear single-stranded fragment.

Several computer programs are available which assist in the calculation of optimal annealing temperatures, GC-contents, secondary structures, primer-dimer formation and other properties of primers (e.g. GENE RUNNER 3.00, Hastings Software, Inc., 1994).

Even when the above guidelines are fully considered, primer design is, however, not straightforward. A given amino acid may be encoded by different triplets due to the degenerate nature of the genetic code (see Table 2.2). As a consequence, a mixture of primers (‘degeneration’) instead of a primer with a defined sequence is required. The degeneracy of the nucleotide triplet encoding a certain amino acid is an important issue in the design of primers: degenerate primers enable the amplification of related but distinct nucleic acid sequences as well as of targets for which only amino acid sequences are available (e.g. from protein sequence data or from multiple alignment of related sequences from different organisms). Obviously, the use of protein sequences containing amino acids with little degeneracy is desirable because it provides the greatest specificity. No general rules can be given for the degree of degeneracy that allows successful amplification. Even 1024-fold degenerated primers have been shown to work well in PCR (Knoth et al., 1988).

Several approaches can be considered for increasing the specificity of the amplification using degenerate primers (Kwok et al., 1994): (i) the primer pools may be synthesized as subsets where one contains either a G or C at a particular position, whereas the other contains either an A or T at the same position; (ii) the degeneracy of the mixed primer may be reduced by considering the codon bias for translation. For instance, codons having an A in the third position (e.g. CTA or TTA for leucine) are used only rarely (Table 2.2); (iii) degeneracy at the 3′-end of the primer should be avoided, because single base mismatches may obviate extension; (iv) the inclusion of deoxyinosine (I) at some ambiguous positions may reduce the complexity of the primer pool. Several experiments have suggested that deoxyinosine might be an ‘inert’ base (Martin et al., 1985) and its presence in an oligonucleotide sequence probably will not cause any disturbance in DNA duplex formation or result in destabilization of the duplex. However, pairing of dI with the four different bases is not equal: dI:dC >> dI:dA > dI:dT = dI:dG (Ohtsuka et al., 1985). Furthermore, it has to be taken into account that deoxyinosine oligonucleotides cannot be used with some DNA polymerases, such as Vent or Pfu (Knittel and Picard, 1993).
Table 2.2. Codon usage in ascomycetous filamentous fungi.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Codon</th>
<th>A. nidulans&lt;sup&gt;b&lt;/sup&gt;</th>
<th>A. niger&lt;sup&gt;b&lt;/sup&gt;</th>
<th>N. crassa&lt;sup&gt;b&lt;/sup&gt;</th>
<th>T. reesei&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
<td>Number</td>
<td>%</td>
<td>Number</td>
</tr>
<tr>
<td>Gly</td>
<td>GGG</td>
<td>893</td>
<td>15</td>
<td>178</td>
<td>8</td>
</tr>
<tr>
<td>Gly</td>
<td>GGA</td>
<td>1323</td>
<td>22</td>
<td>386</td>
<td>16</td>
</tr>
<tr>
<td>Gly</td>
<td>GGT</td>
<td>1710</td>
<td>28</td>
<td>863</td>
<td>36</td>
</tr>
<tr>
<td>Gly</td>
<td>GGC</td>
<td>2091</td>
<td>35</td>
<td>944</td>
<td>40</td>
</tr>
<tr>
<td>Glu</td>
<td>GAG</td>
<td>3139</td>
<td>59</td>
<td>1019</td>
<td>71</td>
</tr>
<tr>
<td>Glu</td>
<td>GAA</td>
<td>2221</td>
<td>41</td>
<td>414</td>
<td>29</td>
</tr>
<tr>
<td>Asp</td>
<td>GAT</td>
<td>2348</td>
<td>47</td>
<td>691</td>
<td>41</td>
</tr>
<tr>
<td>Asp</td>
<td>GAC</td>
<td>2642</td>
<td>53</td>
<td>991</td>
<td>59</td>
</tr>
<tr>
<td>Val</td>
<td>GTG</td>
<td>1309</td>
<td>23</td>
<td>498</td>
<td>27</td>
</tr>
<tr>
<td>Val</td>
<td>GTA</td>
<td>532</td>
<td>9</td>
<td>83</td>
<td>5</td>
</tr>
<tr>
<td>Val</td>
<td>GTT</td>
<td>1597</td>
<td>28</td>
<td>433</td>
<td>24</td>
</tr>
<tr>
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<sup>a</sup>Three-letter code.

<sup>b</sup>Total number of genes used in the derivation of this data: *Aspergillus nidulans*, 146; *Aspergillus niger*, 57; *Neurospora crassa*, 208; *Trichoderma reesei*, 32.

### 2.2.4 Other reaction components

The PCR reaction requires magnesium-chelated dNTPs in a pH-stabilized environment. The concentration of each of the nucleotides should be in the range of 50–200 µM and should be balanced. Differences in concentration of the nucleotides may cause misincorporation and thereby decrease yield and
fidelity of the PCR (Ehlen and Dubeau, 1989). Most suppliers of thermo-
stable DNA polymerases also supply the respective buffers, and hence their
optimization is usually no problem. Changing the Mg^{2+}-concentration from
1 to 10 mM can have dramatic effects on the specificity and yield of an
amplification. The presence of EDTA or other metal ion-chelating agents,
which are used in some DNA extraction procedures (Möller et al., 1992) and
may therefore be carried over, lowers the effective concentration of Mg^{2+}. An
excess of dNTPs has the same effect. The concentration of metal chelators
and total dNTPs should therefore be taken into account when determining
the final Mg^{2+} concentration required for PCR.

The use of 1–10% (w/v) dimethylsulphoxide (DMSO) in the PCR assay
has been recommended (Scharf et al., 1986; Chamberlain et al., 1988),
because it lowers the melting temperature of the dsDNA and hence facilitates
strand separation. This may be particularly important in the denaturation of
GC-rich DNA and help to overcome difficulties caused by DNA secondary
structures (Hung et al., 1990). However, the use of DMSO is not generally
recommended, as it apparently increases the solubility of the mineral oil
(frequently used as a protectant against evaporation) in the aqueous phase
and thereby inhibits DNA polymerase (Linz, 1991). An alternative to the
mineral oil layer is the use of a thermocycler with an integrated heated lid.
Use of formamide (1.25–10%) can also facilitate primer–template annealing
reactions and lower the denaturing temperature of DNA (Sarkar et al.,
1990).

Glycerol is also frequently included in the reaction system (5–20%,
w/v), because it may improve the yield of the amplicon by stabilizing the
DNA polymerase (Instruction Manual for Pfu DNA Polymerase; Stratagene,
La Jolla, California). A similar stabilization can also be achieved by the
addition of bovine serum albumin (10–100 µg/ml), which has the further
advantage of binding fatty acids and phenolic compounds which may inhibit
the PCR (Pääbo et al., 1988).

2.2.5 Cycling parameters

In a typical PCR reaction, the double-stranded DNA is denatured by
heating the sample to 94–98°C. The primers are allowed to anneal at the
calculated annealing temperature (typically 40–60°C) followed by heating
to the extension temperature (depending on the thermostable DNA
polymerase used, 68–76°C). This cycle is repeated 25–40 times. Normally,
these cycles are preceded by an initial denaturation step of 1–3 min and
followed by a final extension step at the chosen extension temperature
for 5–10 min. To avoid extension of unspecifically annealed primers at
lower temperature while setting up the PCR mixture, a ‘hot start’ is
recommended. This means that the enzyme is added to the reaction
mixture after the initial denaturation step only. ‘Hot start’ has dis-
advantages, however, in that it is laborious when handling many samples
at the same time, and carries the risk of introducing contamination. As an alternative to ‘hot start’, Taq antibodies can be added to the reaction mixture. The antibody inhibits Taq polymerase during the setting up of the reaction mixture only because it is subsequently inactivated at the first denaturation step. Denaturation should be as short as possible to avoid inactivation of the thermostable DNA polymerase by prolonged exposure to elevated temperatures. On the other hand, complete denaturation is an absolute requirement for high efficiency PCR. For most applications denaturation times between 30 s and 1 min are recommended. The thermostability of different thermostable DNA polymerases varies considerably: *Pfu* DNA polymerase, for instance, is a highly thermostable enzyme, retaining 94–99% of its polymerase activity after 1 h at 95°C. Unlike Taq DNA polymerase, denaturing temperatures up to 98°C can be used successfully with *Pfu* DNA polymerase to amplify GC-rich regions (Chong et al., 1994; Nielson et al., 1995). The Vent DNA polymerase features a half-life of 6.7 h at 95°C and 1.8 h at 100°C. Taq polymerase has a half-life of only 40 min at 95°C and 10 min at 97.5°C.

The temperature and length of time required for primer annealing depend upon the base composition (GC content), length and concentration of the amplification primers. The melting temperature ($T_m$) of a given primer sequence can easily be calculated by the formula:

$$T_m (°C) = 2 (N_A + N_T) + 4 (N_G + N_C)$$

where $N$ equals the number of primer adenine (A), thymidine (T), guanidine (G) or cytosine (C) bases. Usually, the applied annealing temperature is 5°C below the calculated $T_m$, but this has to be optimized individually. Generally, one annealing step should last for about 1 min.

The extension time depends on the length of the target sequence, the extension temperature and the type of thermostable DNA polymerase used. For Taq and Vent polymerase, extension times of 1 min per kb of expected extension products at 72–75°C are recommended. *Pfu* DNA polymerase needs 2 min per kb at 72°C. Using extension times longer than those calculated is not recommended for DNA polymerases possessing proof-reading exonuclease activity, as this would result in the degradation of primers not yet annealed. Too-short extension times, on the other hand, would decrease the yield of PCR product. To circumvent problems due to the lowered activity of DNA polymerase because of repeated denaturation steps, the extension time can be increased continuously from cycle to cycle. The final extension step is necessary to guarantee that only full length PCR products are obtained. After optimization of all parameters, the optimal number of cycles depends mainly on the starting concentration of the target DNA. Too few cycles gives low product yield, whereas too many cycles can increase the amount and complexity of non-specific background products.
2.3 Amplification from RNA (RT–PCR)

For amplification of RNA, cDNA is first synthesized by reverse transcriptase (RT) and then amplified by PCR as the direct amplification of RNA by commercially available thermostable DNA polymerases is not possible (*Tth* thermostable DNA polymerase has been reported to have reverse transcriptase activity but is not used routinely due to its short half-life; Myers and Gelfand, 1991). In filamentous fungi, RT–PCR is usually applied to generate the corresponding cDNA of a previously cloned genomic fragment used for the confirmation of intron boundaries, and to enable their expression in both bacteria and yeasts.

In a straightforward approach a cDNA fragment may also be directly amplified without previous cloning of the respective genomic clone. This may be necessary if amplification of a desired gene from genomic DNA is unsuccessful because of the presence of an intron in the sequence region used for primer design. Furthermore, the complexity of a reverse-transcribed mRNA pool is lower than that of the complete genome, resulting in a lower background of co-amplified byproducts. This specificity may be further increased by the use of a gene-specific oligonucleotide for reverse transcription of the mRNA and two gene-specific primers for subsequent PCR amplification (also see Section 2.5.3). The oligonucleotide primer for reverse transcription therefore has to be the most 3′-located of the three oligonucleotides.

2.4 RACE: Rapid Amplification of cDNA Ends

The RACE protocol (Frohmann *et al.*, 1988) provides a simple and efficient method for the cloning of cDNA from a gene when the sequence of only a single position in the gene (or protein) is known. The design of the 3′-end primer is based on the predictable sequence of the poly(A)-tail of eukaryotic mRNA (e.g. oligo-dT), and hence individual sequence information is only necessary for the 5′-end. This approach has particular advantages if the sequence of the amino-terminus of a protein can be determined because it bypasses the need to sequence internal peptide fragments of the protein as well (e.g. Marx *et al.*, 1995). The method consists of two steps, described in detail (Fig. 2.1).

The use of oligo-dT primers with a 5′-adaptor sequence containing cleavage sites for several restriction enzymes, preferably enzymes with recognition sites that are found infrequently in genomic DNA such as *Sfi*I, *Not*I, *Mlu*I or *Xho*I, is recommended (McClelland and Nelson, 1987); this facilitates subsequent cloning procedures. The first step consists of a reverse transcription of the mRNA as described earlier. The resulting first strand cDNA is then either purified from RNA by RNase H digestion or used directly for PCR amplification with a gene-specific primer and the oligo-dT primer.
For the amplification of the 5′-end of cDNA, the mRNA is reverse transcribed using a gene-specific primer. The product of the first strand cDNA synthesis is then subjected to homopolymer (poly(A)) tailing, using terminal polynucleotide transferase. This allows the amplification of cDNA by means of a gene-specific primer at the 3′-end in combination with a homopolymer primer complementary to the tail at the 5′-end of the first strand.
strand cDNA. The specificity and efficiency of the amplification reaction can be increased by using a second gene-specific primer. Again restriction enzyme recognition sequences can be incorporated into the primer sequences to facilitate subsequent cloning strategies.

To obtain a full-length cDNA clone, primers for amplification of 5′ and 3′ fragments must be designed to produce overlapping PCR clones. The two independent clones can be combined to generate a complete cDNA clone by one additional PCR step using primers hybridizing at the 5′-end of the 5′-RACE fragment and at the 3′-end of the 3′-RACE fragment, respectively, using both fragments as templates. Because of the overlapping character of both fragments the internal sequences are used as primers in the first cycles to generate a full-length cDNA clone. This full-length product is further amplified by the primers at the extreme 5′- and 3′-ends of the cDNA. Alternatively, classical cloning procedures can be used to join the two incomplete fragments.

2.5 Specific Applications

2.5.1 Ramp PCR

In Ramp PCR the annealing temperature is lowered from cycle to cycle, usually by 2°C during the first few amplification steps (PCR Applications Manual, Boehringer Mannheim, 1995). This can be useful if the annealing temperature of a given primer cannot be exactly determined, for example, because of a high degree of degeneracy. Ramp PCR can also be applied when primers of widely different $T_m$ are used. This is based on the rationale that annealing must be highly specific especially in the first few cycles, whereas later cycles are necessary mainly for further amplification.

2.5.2 Long and accurate PCR (LA-PCR)

Amplification of fragments larger than 5 kb by PCR is usually difficult because of the misincorporation of nucleotides due to premature termination of the extension product (Barnes, 1994a). To overcome this limitation, Barnes employed a mixture of two thermostable DNA polymerases, one that is highly processive, and one exhibiting a 3′ → 5′ exonuclease activity which allows ‘proofreading’ of the product. A study of different combinations of various enzymes determined that a mixture of 16 parts KlenTaq (an exonuclease-free mutant of Taq polymerase) to 1 part Pfu polymerase, which has a 3′-exonuclease activity, could yield PCR products as large as 35 kb. The mixture was termed KlenTaq LA-16 and is commercially available (as are many other optimized combinations of two different thermostable DNA polymerases). The differences between classical PCR and LA-PCR are given in Table 2.3.
2.5.3 PCR with Nested Primers

Even though PCR amplification of a gene with highly degenerate primers should theoretically lead to a million-fold increase in the abundancy of the amplicon over the amount of DNA originally present, the yields may be much lower in practice, and even too low to detect. In many cases the reason for this is the presence of components inhibiting DNA polymerization, which necessitate the dilution of the original DNA solution to a very low level. ‘Nested PCR’ overcomes this problem by performing a second amplification of the accumulated amplicon using primers annealing within the previously amplified product (nested primers; Albert and Fenyő, 1990). Since the first amplification also reduces the nucleic acid complexity, this method enables a high specificity in the second amplification. Sometimes, only a single internal primer in the second PCR may suffice. Design of a nested primer can be based on the addition of as little as three bases to the 3’-end of the first stage primers. Because of this specificity, nested PCR can be carried out with highly degenerate primers (e.g. 8192-fold; Chen and Suttle, 1995).

2.5.4 Inverse PCR

Inverse PCR allows the amplification of DNA regions located outside of a previously characterized sequence (Ochman et al., 1988; Triglia et al., 1988; Silver and Keerikatte, 1989) and thus enables the direct cloning of a full-

<table>
<thead>
<tr>
<th>LA-PCR</th>
<th>Classical PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klen TaqLA-16</td>
<td>Only one enzyme</td>
</tr>
<tr>
<td>pH 9.2</td>
<td>pH 8.3</td>
</tr>
<tr>
<td>16 mM (NH₄)₂SO₄, no KCl</td>
<td>50 mM KCl</td>
</tr>
<tr>
<td>50 mM Tris</td>
<td>20 mM Tris—HCl</td>
</tr>
<tr>
<td>3.5 mM MgCl₂</td>
<td>1–2 mM MgCl₂</td>
</tr>
<tr>
<td>30–40 s 99°C</td>
<td>60 s 95°C</td>
</tr>
<tr>
<td>2 ng λ-DNA</td>
<td>20 ng λ-DNA</td>
</tr>
<tr>
<td>33 µl reaction volume</td>
<td>100 µl reaction volume</td>
</tr>
<tr>
<td>20 cycles</td>
<td>30 cycles</td>
</tr>
<tr>
<td>68°C extension temperature</td>
<td>72°C extension temperature</td>
</tr>
<tr>
<td>33 nucleotide primers</td>
<td>20–22 nucleotide primers</td>
</tr>
<tr>
<td>11–24 min extension (longer at later cycles)</td>
<td>3–10 min extensions</td>
</tr>
<tr>
<td>Hot start or Taq antibody start</td>
<td>Cold start, no antibody</td>
</tr>
<tr>
<td>Filter tips</td>
<td>Non-filter tips</td>
</tr>
<tr>
<td>UVA + 8 MOPa before template</td>
<td>No treatment</td>
</tr>
</tbody>
</table>

a8 MOP, 8-methoxy-psoralen (for inactivation of contaminating DNA).

Table 2.3. Comparison of conditions for LA-PCR and classical PCR to amplify a 35 kb fragment from λ-DNA (Barnes, 1994b).
length (e.g. containing also the 5′ and 3′ untranslated regions) gene. This technique is based on the simple rationale that digestion of a given region of DNA with restriction enzymes, and the circularization of the respective fragments before amplification, allows the use of PCR with primers synthesized in the opposite orientations to those normally used for PCR (Fig. 2.2). DNA is cleaved by restriction enzymes which have no recognition site within the gene fragment of interest. DNA fragments are then ligated under conditions that favour the formation of monomeric circles (Collins and Weissman, 1984). The circularized DNA molecules can then be used for PCR amplification either directly or after precipitation of the template DNA by appropriate salts and ethanol. A similar PCR set-up as described for genomic DNA can be used.

![Fig. 2.2. Application of inverse PCR. The core region is depicted as a jagged line. Filled and open boxes represent the upstream and downstream flanking regions, respectively, and restriction enzyme recognition sites are denoted by triangles. Oligonucleotide primers constructed to anneal to the core region and the direction of DNA synthesis are shown by arrows. Figure from Ochman et al., (1990) with permission.](image)

**2.5.5 Ligation-mediated PCR (LM-PCR)**

Ligation-mediated PCR (LM-PCR; Müller and Wold, 1989) requires the knowledge of only one primer annealing position; the second primer can be defined by ligating a unique DNA linker to it. One of the most important and powerful applications of this method is the rapid cloning of promoters...
and other upstream regulatory elements that control the expression of mRNA.

DNA is digested separately with restriction enzymes which generate blunt ends (e.g. EcoRV, ScaI, DraI, PvuII, SspI) in order to obtain genomic DNA suitable for addition of the unique linkers. Each batch is then separately ligated to the specially designed adaptor. The desired fragment is then amplified in two steps which are based on the same rationale as nested PCR. In the first step, primers annealing to the linker sequence and to the respective genomic sequence are used. In the second step, the linker primer is used together with another which overlaps with the first ‘genomic primer’.

2.6 Cloning of PCR Fragments

Several methods for cloning of PCR-generated DNA fragments have already been published, and only those most routinely used will be described in this chapter. For other methods like ligase-independent cloning (LIC) or uracil DNA-glycosylase (UDG)-treatment of uracil-containing deoxyoligonucleotide primers, the reader is referred to the papers by Aslanidis and De Jong (1990) and Nisson et al. (1991). In addition, several new techniques are currently being developed (e.g. regularly published in *Promega Notes*, Promega, Madison, Wisconsin, USA) which renders it impossible to give a complete list of all available methods.

2.6.1 Incorporation of restriction enzyme sites into deoxyoligonucleotide primers

Incorporation of recognition sequences for restriction enzymes into the primers is probably the most widely used method for cloning PCR fragments. The main advantage of this method is that the fragment can be cloned into a vector construct at precisely the location desired. Any site not contained within the fragment itself can be incorporated into the primer design. When amplifying an unknown sequence, restriction sites should be chosen which occur infrequently in the genome, such as *Not* I, *Sfi*I, *Mlu*I or *Xho*I; in addition, recognition sequences for several restriction enzymes can be incorporated to minimize the chance of cloning an uncomplete PCR fragment because of an internal restriction site.

To guarantee the direction of the cloned insert, different restriction target sites on each primer are usually used. Adding bases to the 5′-end of the primer is the simplest approach and has no effect on the PCR reaction (Scharf et al., 1986; Kaufman and Evans, 1990). When creating a restriction site by addition of sequences to the 5′-end of a primer, it is important to consider that restriction enzymes are endo– (not exo–) nucleases and thus do not usually cleave at the end of a nucleotide strand. Hence the included restriction site needs to be prolonged by the addition of several bases. To achieve efficient cleavage, three
additional nucleotide bp are generally sufficient to act as an annealing clamp. However, some enzymes, for example NotI, need at least 10 bases of double-stranded DNA to restrict at its recognition sequence.

One drawback to restriction enzyme site incorporation is the inability to achieve efficient cleavage. Inhibition of digestion can occur because of the presence of incompatible polymerase buffers and the molar excess of primers left over from the PCR reaction. Some restriction enzymes are inhibited by restriction site-containing primers (Blanck et al., 1995) and therefore, it is often necessary to purify the fragment before restriction enzyme treatment (Table 2.4).

Table 2.4. Activities of restriction enzymes in a PCR mix.

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>% activity in PCR mix</th>
<th>Restriction enzyme</th>
<th>% activity in PCR mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApaI</td>
<td>100</td>
<td>MluI</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Asp700</td>
<td>10</td>
<td>Nael</td>
<td>0</td>
</tr>
<tr>
<td>Asp718</td>
<td>100</td>
<td>Ncol</td>
<td>50</td>
</tr>
<tr>
<td>Avil</td>
<td>30</td>
<td>NotI</td>
<td>0</td>
</tr>
<tr>
<td>BamHI</td>
<td>100</td>
<td>NruI</td>
<td>75</td>
</tr>
<tr>
<td>BbrII</td>
<td>100</td>
<td>PstI</td>
<td>90</td>
</tr>
<tr>
<td>BfI</td>
<td>100</td>
<td>PvuII</td>
<td>&lt;5</td>
</tr>
<tr>
<td>BgiII</td>
<td>0</td>
<td>PvuIII</td>
<td>100</td>
</tr>
<tr>
<td>ClaI</td>
<td>100</td>
<td>SacI</td>
<td>0</td>
</tr>
<tr>
<td>DraI</td>
<td>100</td>
<td>SalI</td>
<td>0</td>
</tr>
<tr>
<td>EcoXI</td>
<td>0</td>
<td>Scal</td>
<td>&lt;5</td>
</tr>
<tr>
<td>EcorI</td>
<td>50</td>
<td>SnaBI</td>
<td>50</td>
</tr>
<tr>
<td>EcoRV</td>
<td>10</td>
<td>SphII</td>
<td>&lt;5</td>
</tr>
<tr>
<td>HindIII</td>
<td>10</td>
<td>SspI</td>
<td>0</td>
</tr>
<tr>
<td>HpaI</td>
<td>100</td>
<td>StuI</td>
<td>30</td>
</tr>
<tr>
<td>KpnI</td>
<td>50</td>
<td>Xbal</td>
<td>60</td>
</tr>
<tr>
<td>KspI</td>
<td>0</td>
<td>XhoI</td>
<td>&gt;5</td>
</tr>
<tr>
<td>Msel</td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative activities of restriction enzymes in a PCR mixture (10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl₂, 200 µM dNTPs, pH 8.3 at 20°C) compared with activity under optimal conditions (SuRE/Cut buffer Boehringer Mannheim) (Blanck et al., 1995).

2.6.2 T/A cloning

T/A-cloning relies on the terminal deoxynucleotide transferase activity of some of the polymerases used in PCR. Clark (1988) showed that some DNA
polymerases and reverse transcriptases contain a terminal deoxynucleotidyl transferase (TdT) activity that results in the addition of one or more nucleotides at the 3'-ends of blunt-ended DNA molecules, which is both nucleotide and polymerase specific (Hu, 1993); e.g., Taq DNA polymerase extends a single dG nucleotide if the 3'-terminal nucleotide on the fragment is a dG but adds a dA if the 3'-terminal nucleotide is a dC. A 3'-terminal dT nucleotide results in the non-addition of a dT and the addition of a dA. Fragments ending with a dA had an additional dA added to the 3'-ends with extremely low efficiency by polymerases that were found to have the TdT activity; there appears to be no consistent patterns for which bases were added. Pfu DNA polymerase does not show any TdT activity and so this polymerase cannot be used for amplification of fragments for subsequent cloning.

The vector into which dA-tailed PCR fragments are to be cloned must contain a 3'-T overhanging sequence. This can be obtained either by incubating a blunt-ended vector with Taq DNA polymerase and an excess of dTTP, or by incubating a blunt-ended vector with dideoxythymidine-triphosphate (ddTTP) and terminal transferase (Holton and Graham, 1990); the use of ddTTP ensures the addition of a single dT residue only.

2.6.3 Blunt-end cloning

As with the T/A System, blunt-end cloning does not require the addition of extra bases to the primer sets. PCR-fragments generated by Taq polymerase or any other polymerase adding a non-templated nucleotide at the 3'-end must be treated with Klenow, T4 or Pfu polymerase to generate blunt ends. The vector must also be blunt-ended (e.g., by cutting with EcoRV or SmaI). To improve the efficiency of blunt-end cloning, the use of an optimized blunt-end ligation buffer is recommended. The direction of fragments after blunt-end cloning is usually not known, but this drawback can be circumvented if one of the primers is phosphorylated at the 5'-end, and the other one is not and therefore still contains the 5'-OH. The vector is cut with the first restriction enzyme, then dephosphorylated and afterwards cut with the second restriction enzyme to create two different blunt ends; one with a 5'-OH, the other with a 5'-phosphate. The ligation of the one-sided-phosphorylated PCR-fragment into the one-sided-dephosphorylated vector allows unidirectional cloning.

Religation of the vector can be a major problem with blunt end cloning of PCR-fragments, but this can be avoided by the use of the blunt-end-generating restriction endonuclease SrfI together with an appropriate vector. The recognition sequence of SrfI is an 8 bp palindrome. Since it is very unlikely that such a sequence occurs in the unknown sequence of the gene of interest, SrfI can be added to the ligation mixture where it cleaves any religated vector (Simcox et al., 1991; Liu and Schwartz, 1992).
Table 2.5. Examples of fungal genes cloned by PCR.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reference</th>
<th>Gene name</th>
<th>Primer based on</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>dm</td>
</tr>
<tr>
<td><strong>Aspergillus</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>Inoue et al., 1991</td>
<td>Proteinase A</td>
<td></td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>Ehrlich et al., 1993</td>
<td><em>phyB</em> (phytase gene)</td>
<td></td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>Jarai et al., 1994</td>
<td><em>pepD</em> (subtilisin-like protease)</td>
<td></td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>Van den Brink et al., 1995</td>
<td><em>cprA</em> (NADPH cytochrome P450 oxidoreductase)</td>
<td></td>
</tr>
<tr>
<td><em>A. oryzae</em></td>
<td>Gomi et al., 1993</td>
<td><em>pepA</em> (acid protease)</td>
<td></td>
</tr>
<tr>
<td><em>A. oryzae</em></td>
<td>Lee et al., 1995</td>
<td><em>nucS</em> (nuclease S1)</td>
<td></td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>Sirakova et al., 1994</td>
<td>Metalloproteinase</td>
<td></td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>Reichard et al., 1995</td>
<td><em>pep</em> (aspartic proteinase)</td>
<td></td>
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<tr>
<td><em>A. flavus, A. fumigatus</em></td>
<td>Ramesh et al., 1995</td>
<td><em>mep20</em> (metalloproteinase)</td>
<td></td>
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<tr>
<td><em>A. parasiticus</em></td>
<td>Feng and Leonard, 1995</td>
<td><em>pksl</em> (polyketide synthase)</td>
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<tr>
<td><em>A. parasiticus</em></td>
<td>Cary et al., 1995</td>
<td><em>pecA</em> (polygalacturonase)</td>
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<tr>
<td><strong>Neurospora</strong></td>
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<td></td>
</tr>
<tr>
<td><em>N. crassa</em></td>
<td>Yajima et al., 1991</td>
<td>Photolyase gene</td>
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<tr>
<td><em>N. crassa</em> Mauritaveille</td>
<td>Young and Marzluf, 1991</td>
<td><em>nmr</em> (negative-acting nitrogen control gene)</td>
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<tr>
<td><em>N. crassa</em></td>
<td>Stone et al., 1993</td>
<td><em>gla-1</em> (glucoamylase)</td>
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<tr>
<td><em>N. crassa</em></td>
<td>Tao and Chen, 1994</td>
<td><em>elf-5A</em> (initiation factor 5A)</td>
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<td><em>N. crassa</em></td>
<td>Yatzkan and Yarden, 1995</td>
<td><em>pph-1</em> (2A protein phosphatase)</td>
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<tr>
<td><em>N. crassa</em></td>
<td>Maier et al., 1995</td>
<td>GTP-cyclohydrolase I gene</td>
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<tr>
<td><em>N. intermedia</em></td>
<td>Young and Marzluf, 1991</td>
<td><em>nmr</em> (negative-acting nitrogen control gene)</td>
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</table>

Continued overleaf
<table>
<thead>
<tr>
<th>Organism</th>
<th>Reference</th>
<th>Gene name</th>
<th>Primer</th>
<th>Primer design based on</th>
</tr>
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<tbody>
<tr>
<td>N. sitophila</td>
<td>Young and Marzluf, 1991</td>
<td>nmr (negative-acting nitrogen control gene)</td>
<td>✸dp</td>
<td>ndp</td>
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<td>Penicillium</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>P. chrysogenum</td>
<td>Marx et al., 1995</td>
<td>paf (antifungal protein)</td>
<td>✸</td>
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<tr>
<td>P. chrysogenum</td>
<td>Haas et al., 1995</td>
<td>nre (nitrogen regulatory protein)</td>
<td>✸</td>
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<tr>
<td>P. camemberti/U-150</td>
<td>Yamaguchi et al., 1991</td>
<td>mdlA (mono- and diacyglycerol lipase)</td>
<td>✸</td>
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</tr>
<tr>
<td>Trichoderma</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>T. reesei</td>
<td>Barnett et al., 1991</td>
<td>bgf1 (β-glucosidase)</td>
<td>✸</td>
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<td>T. reesei</td>
<td>Törrönen et al., 1992</td>
<td>xyn1, xyn2 (xylanase I and II)</td>
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<tr>
<td>T. reesei</td>
<td>Strauss et al., 1995</td>
<td>cre1 (carbon catabolite repressor protein)</td>
<td>✸</td>
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<td>T. longibrachiatum</td>
<td>González et al., 1992</td>
<td>egf1 (endoglucanase I)</td>
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<td>T. harzianum</td>
<td>Heidenreich and Kubeck, 1994</td>
<td>paf4 (ornithine-5′-phosphate decarboxylase)</td>
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<td>Others</td>
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<td>Aureobasidium pullulans</td>
<td>Li and Ljungdhal, 1994</td>
<td>xynA (xylanase A)</td>
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<td>Amanita muscaria</td>
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<td>gpd (glyceraldehyde-3-phosphate dehydrogenase)</td>
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<td>Boletus edulis</td>
<td>Kreuzinger et al., 1996</td>
<td>gpd (glyceraldehyde-3-phosphate dehydrogenase)</td>
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<td></td>
</tr>
<tr>
<td>Boletus edulis</td>
<td>Mehmann et al., 1994</td>
<td>BoeCHS1 (chitin synthase)</td>
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<tr>
<td>Botrytis cinerea</td>
<td>Causier et al., 1994</td>
<td>chs1 (chitin synthase)</td>
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<td>Cantharellus ciarius</td>
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<td>CacCHS1 (chitin synthase)</td>
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<td>Ceriporiopsis subvermispora</td>
<td>Rajakumar et al., 1996</td>
<td>lip (lignin peroxidase)</td>
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<td>Cochliobolus carbonum</td>
<td>Scott-Craig et al., 1990</td>
<td>PGN1 (endopolygalacturonase)</td>
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<td>Cochliobolus carbonum</td>
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<td>cv-2, cv-7, cv-19 (CPS, cyclic peptide synthase)</td>
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<td>Cochliobolus carbonum</td>
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<td>alp1 (serin protease)</td>
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<td>Cortinarius odorifer</td>
<td>Mehmann et al., 1994</td>
<td>Cooc1S, Cooc2S (chitin synthase)</td>
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<td>Cylindrocladium</td>
<td>Nikolskaya et al., 1995</td>
<td>cly-1, cly-2, cly-3 (CPS, cyclic peptide synthase)</td>
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<td>Diheterospora chlamydospora</td>
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<td>dhy-1 (CPS, cyclic peptide synthase)</td>
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<td>Elaphomyces muricatus</td>
<td>Mehmann et al., 1994</td>
<td>ElmC1S, ElmC2S (chitin synthase)</td>
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<td>Fusarium solani</td>
<td>Gonzalez-Candelas and Kolattukudy, 1992</td>
<td>pelA (pectate lyase)</td>
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<td>Fusarium oxysporum</td>
<td>Sheppard et al., 1994</td>
<td>Bfam1, Cfm1, Cfm2, Ffam1, Kfam1</td>
<td></td>
<td>(cellulase family-specific proteins)</td>
</tr>
<tr>
<td>Hebeloma crustuliniforme</td>
<td>Mehmann et al., 1994</td>
<td>Hec1S1 (chitin synthase)</td>
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<tr>
<td>Hebeloma mesophaeum</td>
<td>Mehmann et al., 1994</td>
<td>HemHS1 (chitin synthase)</td>
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<tr>
<td>Lactarius deterrimus</td>
<td>Mehmann et al., 1994</td>
<td>LcdHS1 (chitin synthase)</td>
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<tr>
<td>Lactarius deterrimus</td>
<td>Kreuzinger et al, 1996</td>
<td>gpd (glyceraldehyde-3-phosphate dehydrogenase)</td>
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<tr>
<td>Laccaria laccata</td>
<td>Mehmann et al., 1994</td>
<td>LaLHS1, LaLHS2 (chitin synthase)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orpinomyces sp.</td>
<td>Chen et al., 1995</td>
<td>cyb (cyclophilin)</td>
<td></td>
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<tr>
<td>Phanerochaete sordida</td>
<td>Rajakumar et al., 1996</td>
<td>lip (lignin peroxidase)</td>
<td></td>
<td></td>
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<tr>
<td>Physisomyces blakesleeanus</td>
<td>Maier et al., 1995</td>
<td>GTP-cyclohydrolase I gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pleurotus ostreatus</td>
<td>Giardina et al., 1995</td>
<td>pox1 (phenol oxidase)</td>
<td></td>
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<tr>
<td>Russula adulterina</td>
<td>Mehmann et al., 1994</td>
<td>RuuHS1 (chitin synthase)</td>
<td></td>
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<tr>
<td>Rhizopogon vulgaris</td>
<td>Mehmann et al., 1994</td>
<td>RhvHS1, RhvHS2 (chitin synthase)</td>
<td></td>
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<tr>
<td>Tuber uncinatum</td>
<td>Mehmann et al., 1994</td>
<td>TuuHS1 (chitin synthase)</td>
<td></td>
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<tr>
<td>Xerocomus badius</td>
<td>Mehmann et al., 1994</td>
<td>XebCS1 (chitin synthase)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* dp, Degenerate primers; ndp, non-degenerate primers; aa/seq, primer design based on partial amino acid sequences of the purified protein; aa/c, primer design based on one or more amino acid sequences from related proteins from other species; dna/cl, primer design based on one or more DNA sequences from related genes from other species.
2.7 Conclusions

The availability of a range of strategies for PCR cloning has revolutionized the cloning of genes from filamentous fungi. Table 2.5 shows a subset of the data available from Medline summarizing genes cloned by some of the PCR strategies outlined in this chapter. While this list is certainly not complete, we have tried to show representative examples for a wide variety of genes, indicating how strongly PCR has taken over fungal gene cloning. It is also apparent from Table 2.5 that there has been a strong increase in the number of publications in recent years, indicating that PCR cloning has now become an established tool in fungal molecular genetics. In addition (and not shown here), more sophisticated techniques such as differential display and ligation-mediated PCR are being applied increasingly to filamentous fungi. It is anticipated that these advances in methodology will help raise fungal mycology to the status already reached by the molecular genetics of unicellular pro- and eukaryotes.

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3.1 Introduction

Every biological process results from selective expression of the genome. Processes such as cell division and proliferation, differentiation, ageing and response to stress situations are governed by modulation of the expression of specific genes or sets of genes. Studies on differential gene expression have therefore attracted much interest and research effort. Until recently, the isolation of differentially expressed genes has largely relied on differential hybridization (Sargent, 1987) and subtractive hybridization procedures (Lee et al., 1991). These methods are laborious and time-consuming, and usually require relatively large amounts of starting material. Furthermore, their sensitivity is limited, only allowing the detection of relatively abundant mRNAs. In 1992 a new procedure for differential gene expression analysis was described: 'differential display reverse transcriptase–polymerase chain reaction' (DDRT–PCR) (Liang and Pardee, 1992). This method makes it feasible to display the full set of mRNAs in a particular cell type and to compare expression patterns, either with other cell types or with cells subjected to other experimental conditions. DDRT–PCR possesses several advantages over subtractive and differential hybridization procedures. Firstly, it is quicker because probes for Southern and Northern blot analysis as well as partial sequence information of differentially expressed genes may be made available in a few days. Secondly, only a small amount of biological material is required, making this methodology particularly useful when the amount of starting material is limiting (Zimmermann and Schultz, 1994). Thirdly, the application of PCR increases the sensitivity and thereby allows the detection of low abundance mRNAs (Liang and Pardee, 1992). These
features all contribute to the power and utility of this method. Since it was first described, refinements have been introduced and the procedure has been applied in a wide variety of biological systems (reviewed by Liang and Pardee, 1995).

We have applied DDRT–PCR to study gene expression in *Botrytis cinerea*, a ubiquitous fungal plant pathogen causing important economic losses in many agricultural crops (Jarvis, 1977). The aim of this research is to investigate fungal gene expression *in planta* during the interaction of *B. cinerea* with the host plant, tomato (*Lycopersicon esculentum*). We assume that mutual signalling during the interaction results in changes in gene expression both in the fungus (for activation of mechanisms for penetration and colonization of the host tissue) and in the plant (for activation of defence responses). Among the fungal genes whose expression is induced during the interaction, it is likely that some of the genes are essential for pathogenesis. To this end, it was decided to undertake a systematic comparison of gene expression of the fungus *in planta* and during growth in liquid culture. When studying such a system, the limiting factor is the small amount of fungal biomass in the interaction material, particularly during early stages of the infection process. Therefore a highly sensitive method, such as PCR, is required.

In this chapter we summarize the principles of DDRT–PCR and experimental parameters that were considered during the development of the methodology. Furthermore we describe its application to the analysis of *B. cinerea* gene expression *in planta*. Our results illustrate the high sensitivity and versatility of the method.

### 3.2 Principles of DDRT–PCR

The differential display procedure is designed for the identification and isolation of genes that are differentially expressed in various cell populations under defined conditions. It combines techniques that are commonly applied in molecular biological research: reverse transcription, PCR and electrophoresis in polyacrylamide gels (Fig. 3.1). In the first step, a subpopulation of mRNAs is reverse transcribed into cDNA by using an ‘anchored’ 3′ primer, taking advantage of the presence of a poly(A) tail in most eukaryotic mRNAs. The anchored primer consists of a number of thymidine residues (usually 11), which anchor it to the poly(A) tail of the mRNA, plus two additional nucleotides at its 3′-extreme which provide the specificity of the annealing. Such a primer anneals selectively to mRNAs containing two nucleotides complementary to those at the 3′-end of the primer immediately upstream of the poly(A) tail. In total, 12 anchored primers can be designed (12 different combinations of the last 3′ nucleotides omitting thymidine at the penultimate position). Thus, 12 cDNA subpopulations need to be generated if the entire mRNA population is to be analysed. The reduction of template
complexity by using selective anchored primers for cDNA synthesis is an essential step in the procedure.

PCR is subsequently performed on each of the cDNA subpopulations, using the same anchored primer applied in cDNA synthesis in combination
with a small (10-mer) upstream primer. Any cDNA species is suitable for amplification by PCR if the distance between the poly(A) tail and the position at which an upstream primer anneals is smaller than 2–3 kb. For a 5′ primer of arbitrary nucleotide sequence, annealing positions (if present) are distributed randomly upstream of the poly(A) tail. The resulting amplified products represent only parts of a particular mRNA species (3′) and products derived from various mRNAs will differ in length. If PCR is performed in the presence of a labelled nucleotide (usually [α-33P]dATP), the amplified products can be resolved in a polyacrylamide gel and visualized as discrete bands by autoradiography. The band patterns of samples derived from different cell types or from cells cultured in different conditions are analysed in parallel. Fragments of cDNA representing differentially expressed mRNAs can be recovered from the gel and used to further characterize the corresponding genes.

3.2.1 Considerations for experimental parameters

Since it was first described in 1992, the differential display methodology has been applied to a variety of biological systems. Many groups have adapted the original protocol to their particular systems and requirements. Although general guidelines can be given, it is our experience that the optimal conditions must be found by each user when applied to a particular system. Below, we will discuss briefly the main variations that can be considered in each step of the differential display procedure and point out the conditions that we have found most appropriate in our system.

Starting material and reverse transcription
The most critical factor influencing DDRT–PCR analysis is the quality of the starting material. Protocols for RNA isolation yielding undegraded and highly purified RNA should be used (guanidinium thiocyanate-based protocols usually give good results). Both total RNA and poly(A)+-RNA can be used as template for reverse transcription and nearly identical band patterns are obtained (Liang et al., 1993). For simplicity, total RNA has been used in many cases. In our hands, however, better results were obtained using poly(A)+-RNA, in terms of both the number of bands produced and reproducibility.

The original protocol made use of anchored primers with two selective nucleotides for the cDNA synthesis. More recently, protocols have been described which use anchored primers with two selective nucleotides in which the penultimate position is degenerate (Liang et al., 1993), or anchored primers with only one selective nucleotide (Liang et al., 1994). Such primers have allowed a significant reduction of the number of cDNA synthesis reactions required for each RNA sample. However, the use of such primers will generate cDNA populations of higher complexity that serve as templates in the subsequent PCR step, resulting in bands occupying almost every
position of the gel. This makes the analysis of the band patterns more complicated. Additionally, as pointed out by Bertioli et al. (1995), a reduction of template complexity will contribute to an increase in the sensitivity of the DDRT–PCR procedure for any particular cDNA species.

**Polymerase chain reaction**

The experimental conditions used for PCR amplifications of complex templates in the differential display procedure are different from conditions applied in standard PCR applications. In the original protocol (Liang and Pardee, 1992), 13 nucleotide-long anchored primers were used in combination with decamers of arbitrary sequence. Low annealing temperatures in the PCR profile are required for annealing of short primers but will also allow mismatches. This appears to be essential for the priming of at least a fraction of the templates. In our hands, temperatures higher than 42°C resulted in a drastic reduction in the number of bands detected while temperatures lower than 40°C produced bands occupying nearly every position in the lane. It was experimentally determined that the specificity of the amplification increases when the dNTP concentration is decreased from 200 µM to 2 µM (Liang and Pardee, 1992). A low dNTP concentration was also essential for labelling the PCR products to a specific activity high enough for detection on an autoradiogram.

An average of 120 bands in the size range of 100–600 nucleotides are consistently detected per primer combination when applied to a higher eukaryotic system. Assuming that about 15,000 genes are expressed in a higher eukaryotic cell, theoretical considerations indicate that at a confidence level of 0.95 the entire mRNA population is displayed, when 12 anchored primers with two selective nucleotides are used in combination with at least 25 upstream primers (Bauer et al., 1993).

Liskens et al. (1995) reported the use of 5′-extended primers as a means of enhancing the reproducibility of the differential display technique. Primers with a length of 22 nucleotides were used in a PCR profile including a few initial cycles at low annealing temperatures. At low temperatures, these primers behave in a similar way to the decamer primers used in the original differential display protocol, since the sequence at the 3′-extreme determines the priming specificity. Subsequent PCR cycles are performed at higher annealing temperatures, resulting in very efficient amplification of the products obtained during the first cycles. This procedure should improve the signal-to-noise ratio of individual bands. The extended primers can be designed so that they contain a restriction site at their 5′-end, making subsequent manipulations easier.

**Separation and retrieval of bands**

Polyacrylamide gels provide the resolution needed to analyse the complex mixtures of amplified cDNAs after PCR. Denaturing polyacrylamide gels have been used in most applications, although for fragments smaller than 200
nucleotides the band patterns generated are more complex. Many of the bands appear as doublets representing both strands of each fragment as a result of the denaturing conditions. Non-denaturing polyacrylamide gels have been used to reduce the complexity of the band patterns (Bauer et al., 1993). We have found that a non-denaturing system gives reproducible patterns and is particularly useful when very complex cDNA mixtures are to be analysed (Benito et al., 1996).

The bands of interest can easily be recovered from the gel by a simple boiling method (Zimmerman and Schultz, 1994). They should be reamplified in reactions containing higher dNTPs concentrations (20 µM) to produce enough DNA for isolation from agarose gels and subsequent cloning. AmpliTaq has most frequently been the enzyme of choice. Because this enzyme adds a single (non-encoded) adenosine to the 3′-end of most of the reaction products, linearized vectors containing 3′-T overhangs are commonly used to clone the PCR products. Cloning strategies based on blunt-end ligation of PCR products are also used. The cloned fragments can be used as probes in Northern and Southern blot analyses for the isolation of the full length cDNA and genomic copies of the gene from libraries. Furthermore, the sequence of the cloned fragments can yield information about the nature of the differentially expressed mRNAs that were detected.

**Reproducibility and reliability**

The DDRT–PCR procedure generates highly reproducible band patterns with the same RNA sample. More than 95% of the bands are detected in common in duplicate reactions and most of the differences observed concern the intensity of the bands (Bauer et al., 1993). To maximize the reproducibility and accuracy of the method in a given experiment, the samples to be analysed should preferably be processed in parallel and in an identical manner. This must be done for every step in the procedure, from RNA isolation to electrophoresis. The same buffers and reagents and, whenever possible, the same batch of enzymes should be used for the entire procedure.

In spite of all the precautions taken, false-positive bands are frequently detected. These include differential bands which fail to detect any mRNA on Northern blots or bands representing non-induced mRNAs. These bands are usually non-reproducible and can be generated as a result of the large number of PCR cycles at low stringency temperatures (Liskens et al., 1995). Performing the analysis of every RNA sample in duplicate may help to discriminate between true- and false-positive bands (Liang et al., 1993). Another source of false-positive bands is the presence of contaminating chromosomal DNA in the RNA sample co-purified during the extraction procedure. This is less of a problem when poly(A)+-RNA is used instead of total RNA for the DDRT–PCR analysis. A DNaseI treatment can be included in the protocol to remove traces of chromosomal DNA (Liang et al., 1993). However, this extra treatment may lead to partial RNA degradation, since DNaseI preparations are never completely free of RNase activity.
3.3 Principles of Differential Display in a Plant–Fungal Pathogen Interaction

We have applied the DDRT–PCR methodology to the analysis of differential gene expression in a complex plant–fungus interaction system, *Lycopersicon esculentum*–*B. cinerea*. Since two organisms are present in the interaction, cDNAs from both will be detected by differential display (Fig. 3.2). Most of these cDNAs represent fungal or plant genes which are constitutively expressed. Their origin can be discriminated if the expression pattern of both organisms displayed during the interaction is compared with the expression pattern of the fungus grown *in vitro* and with the expression pattern of a non-infected tomato plant. Interaction-specific cDNAs are also detected which represent either fungal genes induced *in planta* or plant genes induced in response to the pathogen. Both categories can be discriminated if samples from control infections with a second pathogen are included in the comparative gene expression analysis.

3.3.1 The pathosystem *B. cinerea*–tomato

To investigate the infection process of *B. cinerea* on detached tomato leaves we first established a standardized inoculation procedure. Experimental conditions were designed that determine high infection efficiency by inducing high germination rates of conidia on the leaves and synchronicity of the infection in different lesions. Synchronous infection is essential for a successful temporal analysis of fungal gene expression *in planta*. Under the experimental conditions used, the first symptoms are detectable 20 h post-inoculation (h.p.i.). At this time-point small necrotic lesions start appearing over the leaf surface. The lesions become darker during the following 48 h but neither the number of lesions nor their size increases. At 72 h.p.i., about 1–5% of the total number of lesions start to expand and from these lesions the fungus is able to colonize the whole leaf. At about 120–140 h.p.i. total leaf necrosis is observed and the fungus sporulates on the surface of the necrotized plant tissue. We analysed gene expression of the fungus at two different stages of the infection process: at 16 h.p.i. when no symptom is yet visible but the fungus has already penetrated the host cells, and at 72 h.p.i. at the onset of the formation of spreading lesions.

3.3.2 Sensitivity of the DDRT–PCR procedure

In order to determine whether it is possible to detect fungal gene expression *in planta* at these time-points, we estimated the proportion of fungal biomass in leaves during the infection process over time. *B. cinerea* was inoculated on tomato leaves which were sampled at different times after inoculation. Equal amounts of RNA extracted from these samples were electrophoresed, blotted and hybridized with a probe derived from the *B. cinerea* β-tubulin (tubA) gene,
which is assumed to be expressed constitutively (E.P. Benito and J.A.L. van Kan, unpublished results). The intensities of the signals detected at different time-points were compared with the intensity of the signal obtained on RNA extracted from a *Botrytis cinerea* liquid culture *in vitro*. This intensity ratio provides an estimation of the proportion of fungal RNA/interaction RNA, and therefore of fungal biomass in the interaction material, during the infection. As shown in Fig. 3.3A, this proportion is about 3–5% at the time-points analysed.
Using DDRT–PCR, is it possible to detect and discriminate fungal mRNAs within such a complex mRNA mixture containing only 3–5% of fungal mRNA? We investigated this question by performing a reconstruction experiment. mRNA from *B. cinerea* in vitro liquid cultures and from healthy tomato leaves were reverse transcribed using anchor primer RT3. The plant
cDNA and serial dilutions of the fungal cDNA were mixed and PCR was performed. As shown in Fig. 3.3B, most of the bands detected using cDNAs from healthy leaves (lane 1:0) and cDNAs from B. cinerea grown in vitro (lane 0:1) as template in PCR, are also detected in a single lane when equal amounts of both cDNAs are mixed and used as template (lane 1:1). When cDNA from B. cinerea grown in vitro is diluted in relation to cDNA from healthy leaves, the intensity of the bands representing B. cinerea cDNAs decreases with increasing dilution, while the intensity of the bands representing plant cDNAs remains constant (lanes 1:1/10–1:1/100). Even at the highest dilution tested, several B. cinerea cDNA-derived bands are still detectable. In the sample with the 1/30 dilution factor a large proportion of bands representing B. cinerea cDNAs are detected. Since the amount of fungal cDNA in this sample is comparable to that from an infected leaf in which 3% of the interaction mRNA is from B. cinerea, we assumed that the DDRT–PCR procedure was sensitive enough to apply to our experimental system. However, it must be noted that the weakest bands detected in the B. cinerea in vitro-grown sample are not detected in the reconstruction sample, suggesting that some fungal mRNAs expressed at a low level in planta may be overlooked.

3.3.3 Analysis of the expression pattern in the B. cinerea–tomato interaction

We initiated the analysis of B. cinerea in planta gene expression. As mentioned above, samples from control interaction systems are required to discriminate fungal genes induced in planta from plant genes induced in response to the pathogen. A useful control interaction in our experimental approach should mimic as much as possible the plant defence response induced by B. cinerea. For this purpose, we used two different pathogens: the fungus Phytophthora infestans and tobacco necrosis virus (TNV). On tomato leaves, P. infestans induces necrotic spots that appear in the centre of water-soaked areas at about 50–55 h.p.i., whereas TNV induces small necrotic spots at 70 h.p.i. For both control pathogens, samples were collected shortly before symptoms became detectable: 40 h.p.i. for P. infestans-infected leaves and 60 h.p.i. for TNV-infected leaves.

A first set of experiments was performed in order to determine the best conditions for DDRT–PCR analysis and to test the validity of our experimental approach on a limited number of samples: B. cinerea-infected tomato leaves collected at 16 h.p.i.; B. cinerea cultured in vitro; non-infected tomato leaves; and P. infestans-infected tomato leaves.

Figure 3.4A shows a representative example of a differential display gel obtained. In all experiments duplicate reactions were included of the B. cinerea–tomato interaction samples (using cDNAs derived from two independent reverse transcriptase reactions) to reduce the number of false-positive bands. An average of 100–120 bands were detected in lanes from
plant cDNA samples, while 70–80 bands were detected in samples derived from *B. cinerea* grown in vitro. About 15–20% of the latter bands were also detected in the *B. cinerea*–tomato interaction lane for most of the primer combinations. Figure 3.4B shows in detail the patterns obtained with a given primer combination. In addition to the bands representing constitutively

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**Fig. 3.4.** (A) Example of a ‘differential display’ gel. mRNA samples were reverse transcribed with anchored primer RT2 and PCR amplifications were performed using the same anchored primer in combination with eight different upstream primers (P1–P8). For each primer combination samples are as indicated in (B). (B) Detail of the band patterns obtained by ‘differential display’ with primers RT2 and P1 using cDNAs derived from mRNAs obtained from: I.P.i., *P. infestans*–tomato interaction 40 h.p.i.; I.B.c., *B. cinerea*–tomato interaction 16 h.p.i. (duplicate reactions, 16 h-1 and 16 h-2); L.e., healthy tomato leaves; B.c., *B. cinerea* grown in vitro. The arrows indicate interaction-specific bands. Reproduced from Benito et al. (1996) by permission of Kluwer Academic Publishers.
expressed mRNAs from *B. cinerea* and tomato, novel bands are detected in samples derived from *B. cinerea*-infected tomato leaves (indicated by arrows). Some of these bands are also detected in samples from *P. infestans*-infected tomato leaves (arrows 2 and 3) and probably represent plant defence genes induced in response to both pathogens. Other bands are apparently specific to the *B. cinerea*–tomato interaction and are candidates to represent *B. cinerea* mRNAs induced in planta (arrow 1).

In view of the fact that interaction-specific bands were detected, the analysis was extended including mRNA samples derived from *B. cinerea*-infected tomato leaves collected 72 h.p.i. and from TNV-infected tomato leaves as a second control interaction system. In total, 52 primer combinations were used (two anchored primers and 26 upstream primers) in these experiments and 22 *B. cinerea*–tomato interaction-specific fragments were detected. About 4000 *B. cinerea* cDNA fragments derived from genes expressed *in vitro* were displayed, of which 700–800 were also detected in interaction samples. Since we have examined only two out of the 12 possible cDNA subpopulations, the entire analysis should yield about 4000–5000 *B. cinerea* cDNA fragments derived from genes expressed *in planta*.

### 3.3.4 Further characterization of bands of interest

Further characterization of ‘differential’ bands detected by DDRT–PCR includes confirmation of differential expression by Northern blot analysis and cloning to obtain sequence information. In an interaction between two organisms, the origin of the differentially displayed bands needs to be determined. To this end, these bands were recovered from the gel, reamplified, labelled and hybridized to a Southern blot containing *Hind*III digests of genomic DNA from tomato and from *B. cinerea* (we have found that labelling by PCR is a much more effective procedure for labelling small DNA fragments such as those obtained in DDRT–PCR analysis than labelling by ‘random priming’). Four fragments hybridizing with genomic DNA of *B. cinerea* were selected for further analysis. It is commonly observed in differential display analysis that single bands represent several molecular species of the same size (Callard *et al.*, 1994; Li *et al.*, 1994). To test this possibility the fragments hybridizing with *B. cinerea* genomic DNA were cloned and inserts from individual plasmids were used to confirm the hybridization pattern on Southern blots. Only inserts reproducing the hybridization pattern originally obtained were used for *in planta* gene expression analysis on a time-course Northern blot. Two of the four fragments tested appeared to be homogeneous while the other two were mixtures of at least two different DNA fragments: only one of the fragments reproduced the initial hybridization pattern whereas the second one hybridized neither to *B. cinerea* nor to plant genomic DNA. Northern blot analysis confirmed differential expression *in planta* for three of the cDNAs detected and these are currently characterized in detail. The fourth one did
not detect any signal on the Northern blot, probably due to a low level of expression.

When large numbers of ‘differential’ fragments are to be tested, alternative screening procedures based on ‘mass’ analysis can be performed (Mou et al., 1994). Duplicate filters containing equal amounts of the cDNA fragments of interest are probed with labelled total genomic DNA to determine the origin of the cDNAs isolated and with labelled cDNA synthesized using mRNAs isolated from the sources under investigation. Many fragments can be processed simultaneously in this way. However, the method has limitations since frequently, as mentioned above, differential bands represent a mixture of DNA sequences. In this situation, positive cDNAs expressed at low levels can be overlooked.

Sequencing of the cloned fragments can provide information about the nature of the fragments detected through database searches. According to the principle of the differential display procedure, the fragments displayed represent the 3′ extreme of the mRNA molecules. Usually the two primers used to amplify a particular fragment are found at the extremes, thereby providing information about the orientation of the fragment. A limitation of the procedure is that the amplified fragments are relatively small and only represent a (largely untranslated) part of the mRNA, thus restricting the usefulness of the sequence information obtained (Sompayrac et al., 1995). Furthermore, fragments often arise as a result of amplification primed by only one of the two primers used in the PCR (Guimarães et al., 1995). Such fragments are likely to represent internal regions of the cDNA molecules. In either case, the reamplified fragments can be used as probes to isolate the full length cDNA and genomic copies of the genes of interest.

3.4 Perspectives

As predicted by Liang and Pardee in 1992, the differential display methodology has found a broad range of applications. The potential of the methodology is enormous. As originally described, the method is only qualitative (Liang and Pardee, 1992), but modifications have been proposed which would make the method semiquantitative, with potential applications in diagnostic procedures (Bauer et al., 1993; Guimarães et al., 1995). Moreover, it is not limited to the comparison of two mRNA samples, like subtraction-based methods. Instead it allows the comparison of multiple mRNA samples, for instance in the analysis of spatial or temporal gene expression (tissue-specific expression, different developmental stages) (Utans et al., 1994; Guimarães et al., 1995). Furthermore, its sensitivity is high, although this is controversial. Bertioli et al. (1995) presented experimental data suggesting that differential display shows a strong bias towards high copy number mRNAs. Guimarães et al. (1995), on the contrary, demonstrated that DDRT–PCR is able to detect low abundance
mRNAs. Our results support the high level of sensitivity of the method, since induced mRNAs were detected within a subpopulation of mRNAs which constitutes only 3–5% of the total infected leaf mRNA population (Benito et al., 1996).

Differential display has become the method of choice to detect and isolate differentially expressed genes in many biological systems, mainly higher eukaryotes. Both basic and applied (clinical and medical) areas of research have benefited from DDRT–PCR in mammalian systems (Utans et al., 1994; Zimmermann and Schultz, 1994; Liskens et al., 1995; Yeatman and Mao, 1995) and several groups have reported its successful application in plants (Goormachtig et al., 1995; Sharma and Davis, 1995; van der Knaap and Kende, 1995; Wilkinson et al., 1995). Our work presents one of the first reports on the application of DDRT–PCR to the analysis of differential gene expression in a lower eukaryote, such as a phytopathogenic fungus. Fungi have been used as model systems in fundamental (genetics, biochemistry) and applied (biotechnology, phytopathology, biomedicine) areas of research in the last decades. Information derived from fungal systems has provided significant contributions to the understanding of more complex higher eukaryotic systems. Undoubtedly, DDRT–PCR will be a useful tool when applied to the analysis of differential gene expression in processes of basic interest like mating, morphogenesis and dimorphism. Appleyard et al. (1995) have demonstrated the potential of the differential display methodology for the isolation of genes of biotechnological interest from a filamentous fungus, Gibberella fujikuroi. Chen et al. (1996) used the method to examine the alteration of gene expression of the chestnut blight fungus (Cryptonectria parasitica) when infected with a virulence-attenuating hypovirus. We have successfully applied this methodology to the analysis of differential gene expression in a plant–fungus interaction and demonstrated that it possesses enough sensitivity to analyse fungal gene expression in planta. Provided the appropriate controls are included, plant defence genes induced by a pathogen can also be identified (Benito et al., 1996), demonstrating the versatility of the methodology. Such a procedure enables the study of several aspects of plant–pathogen interactions. It is foreseeable that the DDRT–PCR methodology will find numerous applications in mycology.

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References


Interpretation of PCR Methods for Species Definition

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4.1 Introduction

The introduction of molecular biological techniques has been a major force in the areas of systematics and population biology of filamentous fungi. Unlike the development of taxonomy with bacteria and yeasts, biochemical techniques did not become fully established in the systematics of filamentous fungi, and so the changes that brought about the movement from morphological to molecular characteristics have been very significant. The introduction of PCR-based methods has significantly increased the level of activity in fungal systematics. The simplicity of the techniques, coupled with the general use of particular regions of the genome, has resulted in many important advances in our understanding of taxonomic groupings as well as the evolutionary histories and functional properties associated with them. Hawksworth (1991) and Hawksworth et al. (1995) stated that there were 72,000 accepted fungal species, and that this number was growing rapidly; only 17% of these were represented in culture collections. One potential application of PCR-based techniques is their utility in situations where the sample size is small, and so techniques developed for dried specimens and environmental samples provide opportunities to obtain information on the other 83%. Extraction and amplification of dried material from reference collections is now a relatively straightforward procedure (Taylor and Swann, 1994; Savolainen et al., 1995) and provides many opportunities for examining both current and earlier species concepts and variability.

The major PCR-based techniques that have been considered at the species level for fungi are those developed from the rRNA gene cluster and the random amplification of polymorphic DNA. In addition, some workers
have considered other conserved and variable gene regions such as chitin synthase. The literature available for systematic applications of PCR to fungal systematics continues to grow rapidly and it is unlikely that any review can be entirely comprehensive and timely. However, we have attempted to put together, in this chapter, some general examples of recent developments in this area.

4.2 Species Definitions

Species concepts vary considerably across the range of filamentous fungi, and in many cases may be considered unsatisfactory. They have not, however, attracted the same attention as species concepts in some other organisms. In cases where a meiotic stage of the life cycle can be clearly defined, biological species can be described which are reproductively isolated (Jeffrey, 1973). However, this description is not available for the majority of fungi, which historically have been classified into morphological or phenetic species (Hawksworth et al., 1995). In some cases ecological species have been described, based on adaption to a particular niche, or in plant pathology some species have been defined mainly on host disease symptoms or host association. Morphological, ecological and pathological species are all, therefore, defined from phenetic characters, most of which will relate directly to functional and structural attributes. PCR amplification provides information on DNA sequences and so allows the testing of hypotheses about structure and relationships within phenetically defined species.

In some cases, information on DNA sequences may reinforce existing phenetic species groupings, and the species in these cases can be described as polythetic (Hawksworth et al., 1996). However, DNA sequence information can also provide valuable insights into the evolutionary history of phenetic species, and this can be important in species where the major functional characters available relate to some aspect of their environmental niche, such as in ecological species.

4.3 Ribosomal DNA Gene Cluster

The DNA sequences that encode ribosomal RNAs have been extensively used to study the taxonomic relationships and genetic variations in fungi (Bruns et al., 1992; Hibbert, 1992). The ribosomal RNA gene cluster is found in both nuclei and mitochondria, and consists of highly conserved and variable regions which include the genes for the small 5.8S, and large rRNA subunits (White et al., 1990). The fungal nuclear rRNA genes are arranged as tandem repeats with several hundred copies per genome. The conserved sequences found in the large subunit (LSU) and small subunit (SSU) genes have been exploited to study the many relationships among distantly related
fungi (e.g., Gaudet et al., 1989; Bowman et al., 1992a,b; Bruns et al., 1992).

The spacer regions between the subunits, called the internal transcribed spacers (ITS), and between the gene clusters, called the intergenic spacers (IGS), are considerably more variable than the subunit sequences, and have been used widely in studies on the relationships among species within a single genus or among infraspecific populations (Buchko and Klassen, 1990; Spreadbury et al., 1990; Nazar et al., 1991; Taylor and White, 1991; Anderson and Stasovsky, 1992; Baura et al., 1992; Kim et al., 1992; Lee and Taylor, 1992; O’Donnell, 1992; Molina et al., 1993; Erlands et al., 1994, Li et al., 1994; Buscot et al., 1996).

### 4.3.1 Spacer regions

There are now many examples of the use of either RFLP or sequence differences in the different spacer regions for discriminating between closely related species within a fungal genus. Three genera where there have been results that highlight this approach are **Verticillium**, **Rhizoctonia** and **Fusarium**.

### 4.3.2 ITS region

The ITS consist of two non-coding variable regions that are located within the rDNA repeats between the highly conserved small subunit, the 5.8S subunit, and the large subunit rRNA genes. The ITS region is a particularly useful area for molecular characterization studies in fungi for four main reasons: (i) the ITS region is relatively short (500–800 bp) and can be easily amplified by PCR using universal single primer pairs that are complementary to conserved regions within the rRNA subunit genes (White et al., 1990); (ii) the multicopy nature of the rDNA repeat makes the ITS region easy to amplify from small, dilute or highly degraded DNA samples (Gardes and Bruns, 1993); (iii) the ITS region may be highly variable among morphologically distinct species (Gardes and Bruns, 1991; Gardes et al., 1991; Baura et al., 1992; Chen et al., 1992; Lee and Taylor, 1992; Gardes and Bruns, 1993) and so ITS-generated RFLP restriction data can be used to estimate genetic distances and provide characters for systematic and phylogenetic analysis (Bruns et al., 1991); and (iv) PCR-generated ITS species-specific probes can be produced quickly, without the need to produce a chromosomal library (Sreenivasaprasad et al., 1996) and many researchers have selected sequences from the ITS region to develop species-specific probes because the sequences occur in multiple copies and tend to be similar within and variable between fungal species.

PCR-amplified rRNA ITS sequences have been used for the characterization, identification and detection of **Verticillium albo-atrum** and **V. dahliae** (Nazar et al., 1991). In this study the identification of distinct clusters of non-homologous nucleotides in both the ITS1 and ITS2 regions enabled the design of specific primers that provided a reliable identification/detection
method of these two important plant pathogens (Nazar et al., 1991). The same principle was used by Moukhamedov et al. (1993) who used sequences from amplified regions of the 5.8–28S ITS regions to differentiate V. tricorpus from other species of Verticillium. The 5.8S sequences were found to be conserved among these species of Verticillium, but ITS regions of V. tricorpus were sufficiently distinct to allow species-specific primer sets to be constructed which allowed the identification of V. tricorpus from both isolates in culture and from infected potato stems. The PCR-based protocols developed by these workers also permitted the quantification of the pathogen in diseased field plants.

The genus Rhizoctonia consists of a taxonomically diverse group of species that differ in many significant features, including their sexual and asexual stages (Sneh et al., 1996). Within the important phytopathological species R. solani, further intra-specific groups have been designated on the basis of anastomosis (anastomosis groups; AGs). Anastomosis grouping is a convenient way of classifying isolates of the species. However, the process is time-consuming and positive results can be difficult to detect; in addition, misidentification can occur due to the varied frequency of hyphal anastomosis behaviour. The rRNA genes in Rhizoctonia have been examined in relation to the different AG-types in order to develop diagnostic protocols, and to evaluate the characters for phylogenetic relationships (Cubeta et al., 1996). Originally, RFLP analysis of nuclear rDNA was undertaken with probes and Southern blotting (Jabaji-Hare et al., 1990; Vilgalys and Gonzales, 1990), and more recently, PCR-amplified rRNA has been found to be useful in examining the genetic relatedness within different AGs of R. solani and binucleate species of Rhizoctonia (Kanematsu and Naito, 1995; Liu et al., 1995; Vilgalys and Cubeta, 1994; Hyakumachi et al., 1998). One example of these studies is the restriction analysis of amplified ITS1, ITS2 and 5.8S rDNA regions of several R. solani subgroups representing AG1 and AG2 undertaken by Liu and Sinclair (1992, 1993). These workers identified six subgroups within AG1 and five within AG2 on the basis of their ITS-RFLPs. Recently, Liu et al. (1995) constructed restriction maps from amplified products by digestion of the 18S, ITS1, ITS2 and 5.8S rRNA regions of 25 R. solani subgroups. However, at this time most subgroups within R. solani remain largely unresolved. Boysen et al. (1996) used an asymmetric PCR technique on the ITS1, ITS2 and 5.8S rDNA regions with nine AG4 R. solani isolates. These data were used in a phylogenetic analysis which identified three subgroups within AG4. Mazzola et al. (1996) developed species-specific primers for the detection of R. oryzae by comparing ITS1 and ITS2 sequences from R. oryzae and R. solani AG1, -5, -6 and -8. These primers were specific to R. oryzae but not to R. solani or binucleate species. This technique was then used for the detection of R. oryzae from infected wheat tissues and to differentiate the infection caused by R. solani AG8 on the same host plant. Hyakumachi et al. (1998) studied the genetic diversity among three subgroups of R. solani (AG-2-2, IIIB, IV and LP) using a cloned rDNA
probe and RFLP of rDNA-ITS regions. They found that these AG subgroups could be differentiated from the RFLPs of the amplified ITS regions.

The genus *Fusarium* is heterogeneous and the identification of individual species is based on morphological or biochemical criteria which can in some cases be difficult and confusing. Edel *et al.* (1996) differentiated several strains of *F. oxysporum* at the species level by RFLP analysis of a region of ITS and a variable domain of the 28S rDNA. Recently, Schilling *et al.* (1996) evaluated sequence variation in the ITS regions of *F. avenaceum*, *F. culmorum* and *F. graminearum* in order to distinguish between the three species. They found that the ITS sequences of *F. culmorum* and *F. graminearum* were not polymorphic enough to allow the construction of species-specific primers; however, sufficient sequence variation was found in the ITS1 and ITS2 regions of *F. culmorum* and *F. graminearum* to distinguish them from *F. avenaceum*.

### 4.3.3 IGS region

In contrast to the ITS region, fewer studies have considered the IGS region. Arora *et al.* (1996) used RFLP derived from the IGS, located between the rRNA gene clusters, to determine variability within the species *Verticillium chlamydosporium* and other closely related species. They found that there was, in general, a low level of heterogeneity in this region within species, and that distinct IGS types could be associated with particular species. The IGS region has also been investigated in *Fusarium* and Appel and Gordon (1995) found heterogeneity in this region in *F. oxysporum* on the basis of RFLPs of the PCR amplified product. Further studies on the IGS region have been undertaken with *Pythium ultimum*, where length heterogeneity was found to be due to subrepeat arrays (Klassen and Buchko, 1990). Similar long subrepeats have also been reported in the IGS from *Puccinia graminins*, *V. albo-atrum* and *V. dahliae* (Kim *et al.*., 1992; Morton *et al.*., 1995). In *P. ultimum* heterogeneity in the number of conserved subrepeats was found between isolates of the species (Klassen and Buchko, 1990; Buchko and Klassen, 1990), whereas in the *Verticillium* species there was some within-isolate variation of the less conserved subrepeats, but their overall structure and organization could be used to distinguish between the species (Morton *et al.*., 1995).

### 4.3.4 Identification and diagnosis

As already mentioned, the rDNA ITS regions have been examined from a wide range of fungi in addition to the three examples given above, and one of the most common taxonomic uses of the information obtained has been the development of species-specific probes and primers. The region has, for example, been used in the taxonomy of *Oomycetes*, where restriction
patterns from amplified ITS and SSU products have been used to differentiate species of *Pythium* (Chen, 1992; Chen et al., 1992). Levesque et al. (1994) also used a DNA fragment from the ITS region to develop a species-specific probe for *P. ultimum* for routine identification. In addition, within the *Oomycetes*, Lee and Taylor (1992) have sequenced the ITS1 region from three species of *Phytophthora* and synthesized species-specific oligonucleotide probes derived from the non-homologous sequences.

Species-specific primers were also developed by Tisserat et al. (1994) for the diagnosis of turfgrass patch diseases caused by *Ophiobolus herpotricha* and *O. korrae* without the need to first isolate and culture the fungus from the diseased tissues. Kageyama et al. (1997) have used species-specific primers derived from ITS sequences to detect *P. ultimum* in naturally infected seedlings. Bunting et al. (1996) used ITS1 sequences to examine the relationship of *Magnaporthe poae* to other species in the genus that had similar growth or phytopathogenic characteristics. Poupard et al. (1993) used amplified ITS regions in their characterization of *Pseudocercosporella herpotrichoides* isolates. Intraspecific variability in ITS and SSU sequences within the genus *Gremmeniella* was studied by Bernier et al. (1994). A rapid identification method was developed by Vilgalys and Hester (1990) for the identification of *Cryptococcus* species from restriction patterns of ITS and other rDNA regions. Highly sensitive diagnostic assays have also been developed for *Leptosphaeria maculans* based on ITS sequence polymorphism (Xue et al., 1992). PCR–RFLP of ITS has also allowed the discrimination of *Tuber* species (Henrion et al., 1994), the identification of species within the *Gaeumannomyces–Phialophora* complex (Ward and Akrofi, 1994), *Sclerotinia* species (Carbone and Kohn, 1993), *Cylindrocarpon heteronema* (Brown et al., 1993) and *Penicillium* species (Lobuglio et al., 1993). One further example of a genus where extensive use has been made of the ITS region at the species level is *Colletotrichum*. Sherriff et al. (1994) compared a range of isolates of *Colletotrichum* species on the basis of a 886 bp region of the LSU and the ITS2 regions, and were able to use this information to distinguish between individual species. Further extensive species characterization has been undertaken in this genus, leading to the development of a number of species-specific primers (see Sreenivasaprasad et al., 1992).

At a different taxonomic level, universal primers have been developed from rDNA sequences which are specific for major groups of fungi. Gardes and Bruns (1993) designed two taxon-selective primers (ITS1-F and ITS4-B) from the ITS region which were specific to fungi and basidiomycetes respectively. The primer ITS4-B when combined with universal ITS1 primers or with fungal-specific primer ITS-1F, differentiated basidiomycetes from ascomycetes. Primers ITS1-F/ITS4-B were useful for the detection of basidiomycete ectomycorrhizae in rust infected tissues and could be used to study both the distribution of rusts on alternate hosts and ectomycorrhizal communities. In a separate study Hopfer et al. (1993) used fungal-specific primers from the SSU for the diagnosis of fungi in human clinical samples.
The examples detailed here are just a few of the rDNA derived species-specific primers that have been developed to date, and subunit and ITS sequences are likely to continue to be a major tool in molecular identification strategies. Sequence databases are growing rapidly and the approaches discussed here, when combined with sufficient reference data, will provide major insights into the population structures of fungal communities and the detection of fungi from the environment.

4.4 Other Gene-based Approaches

Although much of the recent molecular research into fungal taxonomy has been based on the rRNA gene cluster, other gene sequences have been used both to group fungi at species level and to develop specific probes. The amplification and analysis of many functional genes are dealt with in detail elsewhere in this volume; however, two examples which demonstrate the use of these techniques in taxonomic studies are detailed here. Mehmann et al. (1994) used sequence variation in the chitin synthase genes of some ectomycorrhizal fungi. They found numerous introns within the genes, and used the deduced amino acid sequences to distinguish between species and genera. In another gene-based study, Donaldson et al. (1995) used amplification products from the H3, H4 and β-tubulin genes of Fusarium strains associated with conifers. They used RFLP of these products to group isolates at species level, and also found some heterogeneity within species.

4.5 RAPD and Other Fingerprinting Methods

The random amplified polymorphic DNA (RAPD) fingerprinting assay detects small inverted nucleotide sequence repeats throughout the genomic DNA (Welsh and McClelland, 1990; Williams et al., 1990). In RAPD–PCR, amplification involves only single primers of arbitrary nucleotide sequence. The principle of RAPD assays are discussed in detail by Hadrys et al. (1992) and Tingey and del Tufo (1993). In brief, a single primer binds to the genomic DNA on two different priming sites in an inverted orientation; amplification between these points results in a discrete product. As each primer can be expected to amplify several discrete loci in the genome the final result is generally a profile of amplification products of varying sizes. In addition, at the primer attachment stage in the amplification the annealing temperature is kept low which also encourages a degree of primer mismatching, and increases the potential number of amplification products. RAPD–PCR has many advantages: (i) no prior information for DNA sequence is needed. The protocol is relatively simple and quick and only nanogram quantities of DNA are required to give a PCR product; (ii) the technique is preferred when the genotypes of a large number of species, population or pathotypes
have to be discriminated. RAPD markers can also be used to analyse the
genotypes of fusion products and parents at different taxonomic levels; (iii)
this is a good tool for creating genetic maps (Judelson et al., 1995) and has
proved to be an efficient method for the identification of molecular markers
(Tingey and del Tufo, 1993); and (iv) the technique is suitable for studying
population genetics and has been successfully used to differentiate among
species and strains within species of plants, bacteria, animals and fungi (see
Williams et al., 1990).

RAPD–PCR assays have been used extensively to define fungal popula-
tions at species, infraspecific, race and strain levels. In general, most studies
have concentrated on infraspecific grouping, although others have been
directed at the species level. Some examples of RAPD–PCR at species level
include the production of species-specific probes and primers from RAPD
data for *Fusarium oxysporum* f. sp. *dianthi*, *Phytophora cinnamomii*, *Tuber
magnatum* and *Glomus mosseae* (Dobrowolski and O’Brien, 1993; Lan-
franco et al., 1993, 1995; Manulis et al., 1994). In some RAPD–PCR studies,
band patterns have been used to differentiate both within and between
individual species, as exemplified by species of *Metarhizium* and *Candida*
(Lehmann et al., 1992; Bridge et al., 1997a).

In general, RAPD–PCR has been used most widely to discriminate at an
infraspecific level, particularly in the determination of distinct infraspecific
groups such as anastomosis groups in *Rhizoctonia solani* (Duncans et al.,
1993) and pathogen groups (Crowhurst, 1991; Levey et al., 1991; Guthrie
et al., 1992; Assigbetse et al., 1994; Bidochka et al., 1994; Burmester and
Wostemeyer, 1994; Nicholson and Rezanoor, 1994; Yates-Siilata et al., 1995;
Bridge et al., 1997a; Maurer et al., 1997). Another application of RAPD–PCR
has been in the determination of individual strains within a particular
population, some examples being toxin-producing strains of *Aspergillus
flavus* (Bayman and Cotty, 1993), and in strain authentication in species of
*Trichoderma* (Fujimori and Okuda 1994; Schlick et al., 1994).

Single, simple repetitive primers have been designed to amplify the
microsatellite regions of fungal chromosomal DNA (Meyer et al., 1992;
Schlick et al., 1994; Bridge et al., 1997b). In most applications these primers
have given similar levels of specificity to those seen with RAPD, and so
results have been used to group fungi at infraspecific levels (e.g. Bridge et al.,
1997a). However, in some instances microsatellite-primed PCR has been
used to generate species-specific patterns, and one recent example of this is
the work on morels by Buscot et al. (1996) who found considerable
homogeneity from both mono- and polysporic isolates of individual species.
Further repetitive sequences have been examined for use in fungal system-
atics, including primers derived from the M13 bacteriophage internal repeat,
and the bacterial repetitive extragenic palindromic (REP) and enterobacterial
repetitive intergenic consensus (ERIC) sequences (Hulton et al., 1991;
Versalovic et al., 1991; Meyer et al., 1992). These primers have so far provided
differentiation at the infraspecific level, allowing differentiation of closely
related isolates within single species (Edel et al., 1995; Arora et al., 1996; Bridge et al., 1997b).

4.6 Interpretation of Data

As will already be apparent, a variety of different DNA sequences have been amplified and used in the determination of fungal species. The information available from these studies also varies widely, with some studies presenting full sequences, while others have suggested partially characterized or uncharacterized DNA fragments as species markers (Carbone and Kohn, 1993; Mehamm et al., 1994; Ward and Akrofi, 1994; Faris-Mokaiesh et al., 1996). Most filamentous fungi cannot be grouped into traditional biological species, and the species epithet has been applied at different population levels between and within different genera. As a result, the degree of variability observed within one species will not necessarily be comparable with that found in another. This is not surprising given the large differences in evolutionary age among filamentous fungi, and this can be clearly demonstrated by comparing the variability observed within specific pathogens, which may be recently evolved, such as species of *Colletotrichum* (Sreenivasaprasad et al., 1992), and the variability in presumably long-established saprobic species such as lichen-forming fungi (Gargas and Taylor, 1995). These differences in variability within populations, therefore, make it very difficult to select any one molecular technique as the definitive method for defining fungal species. In practice what is occurring within mycology is a gradual refinement and redefinition of some species with various criteria, in conjunction with other, often phenotypic, properties.

This redefinition will be particularly useful in matching anamorph and teleomorph states, as most DNA sequences can be expected to be consistent between the two states. In cases where an anamorph–teleomorph connection is suspected this may be confirmed by comparative sequence analysis of amplified products as in the placing of *Sporothrix schenckii* within *Ophiostoma* (Berbee and Taylor, 1992), or as has been demonstrated with *Sclerotinia* and *Sclerotium* which showed 98% sequence homology in the ITS1 region of rDNA (Carbone and Kohn, 1993).

4.6.1 Sample size

As the level of variability for any particular molecular characteristic will differ between species, the size of the population considered can be extremely important. In some cases the sample size will be limited by the number of isolates available, and in other instances the cosmopolitan nature of the fungus may require the collection of a wide range of isolates from different hosts and geographic locations. Examples of this latter case are *Fusarium oxysporum* and *Beauveria bassiana*, which are widespread pathogens of
vascular plants and insects, respectively. Within both of these species PCR-based methods have been used to identify host specific populations (Kelly et al., 1994; Neuvéglise et al., 1994; Bentley et al., 1995; Maurer et al., 1997), and these would need to be represented in any definition of the species. PCR-based methods have also been used to define much more restricted populations such as Pneumocystis carinii (Liu et al., 1992), and again sequence differences have been identified between populations from different hosts (Wakefield et al., 1990). The method of analysis used for the data should be appropriate to the sample size and spread, and studies based on many isolates of one species and few representatives of many others may require careful consideration.

4.6.2 RAPD and other fingerprinting methods

RAPD band patterns and other DNA fingerprinting techniques have been used to define some fungal species, and in these studies species-specific bands, or combinations of bands, have been described. In these techniques there is the assumption that bands with identical mobility and staining intensity are of the same or very similar sequence. In general this is not always known, and a further problem is the lack of knowledge as to whether the marker bands consist of coding or non-coding sequences, and as to whether they may represent genes that could be regarded as under selection. There is now some evidence in other organisms that RAPD bands may not be entirely random and that different bands may be homologous (Rieseberg, 1996).

Other features that must be considered when using fingerprinting methods to define species are the potential for random mutation at the fingerprint loci and the sensitivity of the target sequences to crossover and segregation during meiosis (Wu and Magill, 1995). Both of these events have been reported in fungi and may lead to different patterns being obtained from parents and progeny. However, the stability of some fingerprinting methods within some asexually (mitotically) reproducing fungal species has been established, and fingerprinting methods may, in many cases, give species-specific bands or band patterns (Dobrowolski and O’Brien, 1993; Potenza et al., 1994).

4.6.3 Amplification and analysis of the rRNA gene cluster

There have been many applications of the rRNA gene cluster at the species level with filamentous fungi, ranging from simple size comparisons to extensive sequence analyses. Interpretation of these data is, however, not always straightforward as there can be considerable variations between different fungal species. As previously mentioned, the species *Fusarium oxysporum* consists of many different host plant-specific special forms, subdivided into pathogenic races and vegetative compatibility groups. PCR amplification of the ITS region of the rRNA gene cluster, and subsequent RFLP analysis, has
been used within this species to designate subspecific groups (Oullet and Seifert, 1993; Kelly et al., 1994; Bentley et al., 1995). However, within the genus Colletotrichum, species have been delineated on the basis of very small differences in these sequences (Mills et al., 1992; Sreenivasaprasad et al., 1992).

The interpretation of rRNA data in fungal taxonomy is further complicated by the presence of introns within the subunit genes. Many insertion sites have been described in both the large and small subunits and these have been described as possibly important factors in the evolution of fungi (Neuvéglise and Brygoo, 1994; Gargas and DePriest, 1996).

A further important consideration concerns the type and copy number of the rRNA gene cluster itself. While this gene cluster is often described as ‘multi-copy’, in that it may be found in many different parts of the total fungal genome, the different occurrences are not due to recentcopying, and a mutation in one copy will not necessarily be present at all sites. This occurrence is generally accepted to be prevented by concerted evolution, which maintains the homogeneity of the gene cluster and spacer regions (Hillis and Dixon, 1991; Appel and Gordon, 1995). However, there is a small, but increasing, number of publications which suggests that more than one form of the rRNA genes may exist within a single organism. This occurrence has previously been described in a variety of organisms including bacteria, nematodes, insects and mammals (Zijlstra et al., 1995; Kuo et al., 1996; Rainey et al., 1996) and has recently been reported for filamentous and lichen-forming fungi, where some copies of the rRNA gene cluster are suspected to contain sequence differences and can include insertion sequences (DePriest, 1993; Harlton et al., 1995; Sanders et al., 1995). The significance of these events in the interpretation of data for fungal taxonomy has not yet been fully investigated. In addition, the likelihood of detecting such occurrences, given the competitive nature and concentration dependency of the PCR reaction has not been widely considered.

There is an additional single copy rRNA gene cluster located on the mitochondrial DNA of fungi. This region has also been used to develop specific probes and PCR primers for the identification of fungal species (Gardes et al., 1991; Wakefield et al., 1991; Li et al., 1994). As a ‘single copy’ gene this would be free from the complications of different forms being present, and, as mitochondrial DNA is generally transmitted by uniparental inheritance, the gene would be expected to be largely free of recombination effects.

Although the above are important considerations which may be relevant in some areas, many species-specific probes and primers have been designed for fungi from rRNA gene and spacer sequences.

4.7 Numerical Methods for Representing Species

Numerical methods for representing species can be divided very broadly into two groups: those that are used merely to group similar organisms (phenetic);
and those that imply some path or measure of evolution (phylogenetic) (Sneath, 1993).

The development of PCR-based techniques has resulted in the availability of considerable amounts of DNA sequence data that can be used for direct taxonomic purposes. However, the raw data as obtained in these studies can be considered as being phenetic rather than phylogenetic, as the actual data collected are a series of observed (genomic) properties. In order to be correctly termed phylogenetic some measure of time would be required, and this is inferred through the subsequent analyses, rather than directly recorded with the data (Sneath, 1993). As sequence data or band patterns can be treated as phenetic characters, then many of the well-established numerical taxonomy techniques can be utilized to identify or subdivide species groups. Phylogenetic techniques are covered in detail elsewhere in this volume.

Most non-phylogenetic analyses of fungal data have been based on comparing band patterns from RFLP, RAPD or simple repetitive primer approaches (Assigbetse et al., 1994; Pipe et al., 1995; Fungaro et al., 1996; Mordue et al., 1996; Waalwijk et al., 1996). In these analyses, individual bands are considered as equivalent independent characters and so band patterns are usually converted into binary tables. Similarities are derived from established coefficients, and the relationships between isolates are most commonly represented as dendrograms. There are, however, two important factors that must be taken into account in this type of analysis, the first is the relevance of matching negative characters, and the second is the suitability of a hierarchical representation.

### 4.7.1 Similarity coefficients

The majority of dendrograms derived from fungal DNA fragments have been derived with one of three coefficients, the simple matching coefficient, Jaccard’s coefficient and Nei and Li’s genetic distance. The last two of these coefficients do not consider matching negative results, that is, they do not consider the absence of a particular band in two organisms as a similarity, unlike the simple matching coefficient where matching positive and negative results are considered equally (Sneath and Sokal, 1973; Nei and Li, 1979; Bridge, 1992). The validity of considering matching negative characters has been discussed on many occasions (see Sneath and Sokal, 1973; Abbott et al., 1985) but it is perhaps worth further consideration for solely gel-derived data. When numerical methods are not used and gel patterns are compared by eye the operator will use the presence and absence of bands to designate patterns. Therefore, the inclusion of matching negatives within a numerical system may be considered as representative of this, and shape coefficients such as the correlation coefficient have been used in this way in automated gel comparison.
software. However, in taxonomic studies matching negative characters may be acceptable when the characters themselves are relevant and comparison is meaningful. This may be the case where band patterns are derived from RFLP data, and absence of bands can be directly related to sequence information at the restriction site. However, when bands are of unknown origin, such as in RAPD studies, then their relevance cannot be quantified, and it would seem most appropriate to use coefficients that discount matching negatives.

### 4.7.2 Representation

As described above, numerical values are often clustered to give dendrograms, and it is important to consider the appropriateness of this approach. Cluster analysis will always result in the linking of all organisms, no matter how closely or distantly related. One consequence of this is the tendency of markers or distantly related organisms to cluster together as they are similar in that they are different from the rest of the organisms (see Sneath and Sokal, 1973). This situation can arise when the majority of the isolates used in a study belong to a single group, and the remainder are members of different taxa. A further complication can occur with averaging methods such as UPGMA, where the average value obtained from similar values (closely related isolates) is representative of the true similarity, whereas average values obtained from widely different values (distantly related isolates) may not be truly representative (see Abbott et al., 1985). The practical outcome of these occurrences is that cluster analysis is very good at determining relationships within groups and between closely related groups, but may not give an accurate representation of more distant relationships. Where data have been obtained from many members of one or several closely related taxa, cluster analysis will be an appropriate technique. However, when several members of a single species are compared with small numbers of other species, cluster analysis may not be appropriate.

An alternative to cluster analysis is the use of one of the non-hierarchical multivariate techniques such as principal component or principal coordinate analyses. These techniques place organisms on the basis of the overall variability contained within the data, and in contrast to clustering, these techniques provide a more faithful representation of inter-group relationships (see Sneath and Sokal, 1973; Alderson, 1985). Ordination techniques have, therefore, advantages in determining relationships between species, particularly where there may be wide differences in similarity values. Ordination techniques have not been widely used with fungal PCR data, although one of the few such studies published has shown that ordination was superior to cluster analysis in arriving at final species level groupings (Bridge et al., 1997a).
4.8 Conclusions

PCR-based methods offer many new tools that are directly applicable to fungal systematics at the species level. These tools can be used to delimit and to determine relationships among species, either by direct comparison or through phylogenetic analyses. PCR-based methods have given a greater insight into molecular variability within fungi and have highlighted the need to consider carefully sampling strategies and sample sizes, prior to making taxonomic decisions. This insight has also shown that molecular variability is not constant within different fungal species, and levels of both homo- and heterogeneity will vary depending upon the species studied. Perhaps surprisingly, the introduction of PCR-based techniques has not led to a widespread revision of fungal species names and concepts, and in many cases existing species concepts have been reinforced. However, the wide range of molecular heterogeneity found in some species has led to the suggestion that there may be many more ‘cryptic’ and undescribed species within existing collections. Where PCR-based methods will have a very significant impact is in the study of the 83% of known species that do not grow in culture, and the hope is that these techniques may, in future, provide many answers to basic questions in systematics and biodiversity that are currently unanswered.

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5.1 Introduction

Analysis of DNA from lichens was rare prior to the development of PCR-based techniques. In early molecular applications, Blum and Keshevarov (1986, 1992) used total DNA hybridization to estimate the relationship of the genera *Umbilicaria* and *Lasallia*. Isolation and agarose gel electrophoresis of DNA from various cultured mycobionts was carried out by Ahmadjian et al. (1987). With the introduction of PCR (Mullis and Faloona, 1987) during the last decade the situation changed completely and nucleic acids became easily accessible sources of new characters. Today, PCR is an important tool for molecular investigations of mycobiont phylogeny and population genetics.

In this chapter we present an overview of the recent progress of PCR applications in the study of lichen-forming fungi. Because lichens are a symbiotic association of a fungus and an alga, they require special considerations for molecular studies. To date, most molecular work with lichens has been carried out with nuclear ribosomal genes of the fungal partner (mycobiont). This chapter, therefore, focuses on the mycobiont: in the first part we emphasize the specific features of lichens and their significance for DNA isolation and PCR techniques; an overview of phylogenetic approaches to lichens is followed by a section on insertions and introns in ribosomal genes of mycobiont DNA; and the remainder of our contribution concentrates on population studies and miscellaneous PCR applications.
5.2 Characteristic Features of Lichens

As emphasized in the *Dictionary of the Fungi* (Hawksworth *et al.*, 1995), lichens are a biological and not a systematic group of fungi. Hawksworth and Honegger (1994) provided the definition of a lichen as ‘an ecologically obligated, stable mutualism between an exhabitant fungal partner and an inhabitant population of extracellularly located unicellular or filamentous algal or cyanobacterial cells’. Lichens are not the only possible fungal/algal or fungal/cyanobacterial symbioses (Table 5.1) but they signify one of the most successful multibiont associations with a worldwide distribution.

Lichens are particularly remarkable in the diverse morphological features shown by the composite organism. The thalli of certain lichens are among the most complex structures to have evolved in fungi (Douglas, 1994). The fungal partners in lichens are not known to occur in a free-living form, rather they dominate the overall morphology of the vegetative and fertile structures of the symbiotic associations. Nevertheless, there is an interplay between the partners during ontogeny, as different morphologies of thalli may develop by the interaction of a single fungus with cyanobacteria or with green algae (James and Henssen, 1976). Another characteristic feature of almost all lichens is the longevity of the thalli and/or the fruiting bodies. Consequently, the long-term exposure to environmental conditions and the complex organization of thalli may result in a high degree of plasticity of lichens.

The scientific name that is applied to the lichen association strictly refers to the fungal partner alone (ICBN, art. 13.1d; Greuter *et al.*, 1994). Hence,

<table>
<thead>
<tr>
<th>Number of bionts</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Two-biont symbioses</strong></td>
<td></td>
</tr>
<tr>
<td>Mycobiont as inhabitant</td>
<td>Mycophycobioses</td>
</tr>
<tr>
<td></td>
<td>Fungal parasites of algae</td>
</tr>
<tr>
<td>Mycobiont as exhabitant</td>
<td>Lichens</td>
</tr>
<tr>
<td><strong>Three-biont symbioses</strong></td>
<td></td>
</tr>
<tr>
<td>Two photobionts: one mycobiont</td>
<td><em>Cephalodia</em></td>
</tr>
<tr>
<td></td>
<td>Blue-green/green morphotypes</td>
</tr>
<tr>
<td></td>
<td>Algicolous lichens</td>
</tr>
<tr>
<td></td>
<td>Bryophilous lichens</td>
</tr>
<tr>
<td>Two mycobionts: one photobiont</td>
<td>Lichenicolous fungi</td>
</tr>
<tr>
<td></td>
<td>Mechanical hybrids</td>
</tr>
<tr>
<td><strong>Four-biont symbioses</strong></td>
<td></td>
</tr>
<tr>
<td>Three photobionts: one mycobiont</td>
<td><em>Cephalodia</em></td>
</tr>
<tr>
<td>Two photobionts: two mycobionts</td>
<td>Lichenicolous lichens</td>
</tr>
<tr>
<td>Three mycobionts: one photobiont</td>
<td>Fungi on lichenicolous fungi</td>
</tr>
<tr>
<td>Five or more biont symbioses</td>
<td>Mechanical hybrids</td>
</tr>
</tbody>
</table>
the classification and systematics of lichens refer to the relationships among lichen-forming fungi, independent of their symbiotic photosynthetic partners, which have separate algal or cyanobacterial names. The combined association has no name and naming a lichen is synonymous to naming the fungal partner in a lichen association. As a consequence of this, molecular systematic studies have focused on the lichen mycobiont.

5.3 General Remarks on Molecular Work with Lichens

The molecular study of lichen symbioses has been impeded by several problems. Both mechanical isolation of sufficient starting material and axenic cultivation of lichen-forming fungi were cumbersome and impractical approaches for earlier molecular studies. Through the application of PCR, it is now possible to undertake investigations with minute amounts of living or even herbarium material. However, several points must be kept in mind in molecular studies: (i) lichens often grow in dense associations with other individuals or other species. Additionally, they may often be difficult to separate from their substrate. Therefore, lichen material intended for DNA isolations must be examined carefully to avoid exogenous contaminants, such as substrate particles (e.g. bryophytes) or DNA from other fungi (see Petrini et al., 1990). In addition, an important source of undesired DNA may be the occurrence of inhabitant lichenicolous fungi. The algal or cyanobacterial partner is usually not problematic if discriminatory DNA isolation or PCR techniques are used; (ii) lichens are well known for their ability to synthesize high amounts of diverse secondary compounds including polysaccharides. High concentrations of various polysaccharides may inhibit the efficiency of PCR and so isolation techniques must be optimized to eliminate secondary compounds in general and polysaccharides in particular; (iii) PCR anomalies may occur when working with the rDNA of mycobionts. A pair of primers, which works well for many other isolations, may not always amplify the target region even if fresh material is used. Conversely, multiple PCR products may occur even when contamination by other fungal species can be excluded. These phenomena may be due to the presence of optional insertions at priming sites. Because insertions are frequently found in rDNA of mycobionts, they require further consideration and will be described in greater detail later.

5.4 DNA Isolation and PCR Amplification from Lichens

As indicated briefly above, careful dissection of lichen material is important to eliminate DNA of other organisms in general, and that of fungal cohabitants in particular. Both lichenicolous fungi, of which there are about 1000 described species, and lichenicolous lichens, frequently occur on or in
lichens as parasites or commensals (Hawksworth et al., 1995). Microscopic examination prior to dissection of material may confirm the quality of the starting material. Amplification of genomic material from the photosynthetic partner can be circumvented by two approaches. First, fungal material may be isolated mechanically from the lichen thallus, or second, oligonucleotide primers are selected which exclusively amplify fungal DNA.

5.4.1 Mechanical isolation of fungal material

Purely fungal structures are almost always present in lichens. These include hymenia in fruiting bodies, or pycnidia, thalloconidia, rhizines, rhizinomorphs, cilia, medullae, and cortical structures (Fig. 5.1). Successful isolations from podetia (DePriest and Been, 1992), basidiomata (Lutzoni and Vilgalys, 1995a), ascomata (Grube et al., 1995) and rhizines (Crespo et al., 1997a) have been described.

5.4.2 Discriminatory molecular techniques

The most elegant and common approach to amplifying mycobiont DNA is by using fungal-specific oligonucleotides as PCR primers. Using this

Fig. 5.1. Examples of algal-free material used for molecular studies with lichen mycobionts. (A) Decorticated podetium of Cladonia. (B) Chondroid medulla of Usnea. (C) Ascomatal structures. (D) Cortical layers, medulla, and rhizines. (E) Mycobiont cultures.
approach, it is possible to isolate DNA from the total lichen, including the algal/cyanobacterial partner without time-consuming dissection of fungal material. Under appropriate PCR conditions, it is often sufficient to have only one fungal-specific primer in the primer pair. About nine fungal-specific primers suitable for work with lichens have already been designed by comparative analysis of small subunit (SSU) rDNA sequences (Gargas and Taylor, 1992; Gardes and Bruns, 1993; Gargas and DePriest, 1996). These primers also allow selective amplification of fungal internal transcribed spacer (ITS) regions. To date, in the lichen-forming fungi, the nuclear large subunit (LSU) rRNA gene is currently being investigated with fungal-specific primers (P. Clerc, Genève, 1995, personal communication; M. Grube, unpublished work).

Standard DNA isolation protocols which are useful for a wide range of fungi, for example that described by Lee and Taylor (1990), may not be optimal for certain lichens. DNA extraction from lichens must consider the high concentration of polysaccharides present in many species. Armaleo and Clerc (1991) used column purification in their DNA isolation protocols to eliminate PCR and restriction enzyme inhibitors. Their method II also included an enzymatic treatment for the degradation of polysaccharides. More recently, addition of CTAB (cetyl-trimethyl ammonium bromide) to the extraction buffer has proved to be very efficient for DNA isolation from lichens (Armaleo and Clerc, 1995; Crespo et al., 1997a). A protocol described by Grube et al. (1996) relies on the selective affinity of DNA to glass powder and was used to isolate DNA from ascomata.

Optimal yield of isolated DNA is generally achieved with fresh lichen material. However, fresh material is not always accessible and herbarium collections may provide important material for molecular studies. It has been demonstrated that intact DNA can be isolated from mushrooms that have been stored in herbaria for several decades (Bruns et al., 1990; Taylor and Swann, 1994). The DNA degradation rate of herbarium material varies among different lichens and it is usually difficult to get sufficient DNA of Usnea species if the sample is more than 10 years old (data not shown), whereas it has been possible to get considerable amounts of PCR product from DNA of a 30-year-old specimen of Multiclavula mucida (GenBank acc. no. 23542; Gargas et al., 1995a).

Usually, PCR conditions follow manufacturer’s guidelines but they may be optimized depending on primer sequence and target sequence length. Addition of compounds such as dimethyl sulphoxide to the PCR buffer is not considered to improve results. A large number of primers are now available for the PCR amplification of nuclear ribosomal genes of fungi (Gargas and DePriest, 1996; Lutzoni and Vilgalys, 1995a) and a standardized nomenclature for these has been proposed by Gargas and DePriest (1996). Such primers are complementary to the conserved regions of the SSU rDNA, and also to the 5’ region of the LSU rDNA and all have successfully been applied to the PCR amplification of SSU, ITS regions and LSU rDNA of...
lichen-forming fungi. However, 12 of the primers span positions of optional insertions and may fail to amplify if an insertion is disrupting a priming site (Gargas and DePriest, 1996). In such cases, it is advisable to amplify the target region with a different pair of primers. The primers used for PCR may also be applied for the direct sequencing of amplification products. Today, cycle sequencing has become the most popular technique for obtaining sequences from PCR products of lichen-forming fungi, and automated sequencing systems now allow sequence information to be obtained in a relatively short time.

5.5 Molecular Evolution in Lichens

About 13,500 or two-fifths of all ascomycetes occur as lichens and 13 of the 46 orders of ascomycetes include lichen-forming fungi (Hawksworth et al., 1995). Investigation of phylogenetic relationships among lichen-forming fungi is therefore important for a better understanding of fungal evolution in general. The integration of lichen-forming mycobionts into the system of *Eumycota* has been particularly considered by molecular studies on the phylogenetic origins of the lichen symbioses, and an *Eumycota* phylogeny based on parsimony analysis of SSU rDNA sequences has been constructed by Gargas et al. (1995a). In this study five origins of lichenization were detected within the true fungi. Two origins of the lichen state were found in the ascomycetes where the lichen habit arose in groups with both saprophytic and parasitic relatives. Ongoing research is concentrating on a refinement of the *Eumycota* phylogeny by including other lichen groups (Gargas and DePriest, 1996).

Molecular-phylogenetic studies focusing on the SSU rDNA gene have also been carried out at various lower taxonomic levels. These are of special significance in groups where morphological characters are limited, or convergent morphological evolution is expected. Wedin (1996) has presented a phylogenetic hypothesis of four families of *Caliciales*. He demonstrated that representatives of *Sphaerophoridae* form a monophyletic group within the *Lecanorales*. However, the core group of *Caliciales* including *Caliciaceae*, *Mycocacaceae* and *Sphinctriaceae* was not monophyletic. Whereas *Caliciaceae* form a separate monophyletic group within the *Lecanorales*, the two other families appear as a monophyletic group basal to a clade formed by representatives of *Eurotiales* and *Onygenales*. Stenroos and DePriest (1996) provided a phylogeny of stipitate lichens encompassing *Cladoniaceae*, *Cladiaceae*, *Siphulaceae*, and *Stereocaulaceae*. According to their hypothesis, these families form a monophyletic group within the *Lecanorales*. Tehler (1995a, 1995b) and Myllylä and Tehler (1996) carried out a phylogenetic analysis of *Arthoniales* and emphasized that some groups based on morphological data were corroborated by molecular results whereas others were clearly not. Eriksson and Strand (1995) studied the relationships of *Solorina*,
Peltigera and Nephroma and found that Solorina and Peltigera were closely related and should be kept in the same family, Peltigeraceae, whereas Nephroma was distinct and that its placement in a separate family, Nephromataceae, was supported by molecular characters. Marsh et al. (1994) investigated the cladistic relationships within the family Ramalinaceae. The preliminary analyses contributed to the understanding of generic concepts in Ramalinaceae and showed that Dievernia and Ramalinopsis were more closely related to each other than either is to Nieblia.

Søchting and Lutzoni (1996) investigated the SSU rDNA variation in the Teloschistaceae. They showed that the genus Xanthoria is polyphyletic and includes lineages related to Caloplaca subgen. Gasparrina and subfruticose Caloplaca species. A phylogenetic analysis of the Umbilicariaceae was undertaken by Bobrova and Ivanova (1996). According to their hypothesis, Lasallia does not form a sister group to Umbilicaria. The latter appears to be paraphyletic, contradictory to the current morphological concept. Lutzoni and Vilgalys (1995a) and Lutzoni (1996) undertook a phylogenetic analysis of Omphalina, including lichen-forming and free-living species. The sequence data based on ITS and LSU rDNA data suggested that the lichen-forming Omphalina species are a monophyletic group.

With the increasing number of sequences available, several other challenging issues of lichen evolution can be addressed by molecular methods. For example, the taxonomic position of sterile or otherwise poorly understood species can now be evaluated. Hoffmann (1996) analysed DNA from the sterile lichen Normandina pulchella and from co-occurring perithecia referred to as Lauderlindsaya borreri. The latter is sometimes regarded as the fertile stage of Normandina. The preliminary results suggest that Normandina is an ascomycete genus, but different from the lichenicous Lauderlindsaya.

A classic debate in lichenology regarding the reproductive mechanisms and their impact on species evolution has been developed through the discussion about species pairs or ‘Artenpaare’ (Poelt 1970; Culberson and Culberson, 1973; Tehler 1982) and reviewed by Mattsson and Lumbsch (1989). Species pairs are parallel taxa with similar thallus morphology, where one taxon is fertile and the other is sterile, propagating via soredia, isidia or analogous organs. The relationships between species pairs have been investigated using ITS sequences (Lohtander and Tehler, 1996). A cladistic analysis of Dendrographa species suggests that the apomictic strains in this genus originated repeatedly from a fertile primary species (A. Tehler, Salzburg, 1996, personal communication).

Recently, efforts have been made to combine molecular and morphological evidence in lichen phylogenetic studies (Eriksson and Strand, 1995; Lutzoni and Vilgalys, 1995a, b; Tehler, 1995a, b; Lutzoni, 1997). The topology of phylogenetic trees from morphological and molecular data sets may differ significantly and there are several strategies that may be used to obtain single phylogenies. The taxonomic congruence method involves separate
analyses of different data sets and subsequent consensus analysis, whereas the method of total evidence uses both data sets in a combined analysis. Tehler (1995b) discussed the logical difficulties of applying taxonomic congruence and demonstrated the limitations of this method in a phylogenetic analysis of the Arthoniales. Alternatively, Lutzoni and Vilgalys (1995b) proposed that data sets should be analysed separately and be tested if any conflict arose between them. As trees are constructed from samples of data, one important reason for topological differences may be sampling error. If the differences can be explained by sampling error, the morphological and molecular data sets may be combined in phylogenetic analyses. Available tests for evaluating heterogeneity between data sets (e.g. Rodrigo’s test, T-PTP test, and Kishino and Hasegawa test) have been compared by Lutzoni and Vilgalys (1995b). They showed that only the method of Rodrigo et al. (1993) explicitly addressed the question of sampling error.

5.6 Insertions and Introns

It has already been mentioned that PCR results are dependent on the presence or absence of insertions at oligonucleotide priming sites (Gargas and DePriest, 1996). Such insertion sequences, which may differ in length, are found at a high frequency in the ribosomal genes of the mycobiont and they may increase the length of the SSU rDNA by as much as 100%. To date, 17 insertion positions have been described from the nuclear SSU rDNA, and 13 of them are known from lichen-forming fungi (Table 5.2). Seven insertion positions are so far known from the nuclear LSU rDNA, although only one of them is also found in lichen mycobionts. Multiple insertions may be found in a single species, and seven insertions have been found in the SSU rDNA of Lecanora dispersa (Gargas et al., 1995b).

Many of these insertions can be classified as group I introns (subgroup IC; Michel and Westhof, 1990) by sequence analysis. In a pioneering work, DePriest and Been (1992) showed that group I introns have a variable distribution in the rDNA of the Cladonia chlorophaea complex. As detailed later, this variability is of importance for lichen population studies.

When transcribed, group I introns share a common secondary structure and, by their intricate RNA folding, they may act as ribozymes, which catalyse their own excision from their host genes. DePriest and Been (1992) pointed out that the group I introns present in C. chlorophaea do not splice under standard in vitro conditions typically used for self-splicing experiments with other group I introns. This phenomenon is in accordance with a lack of structural elements that are usually found in self-splicing introns (Jaeger et al., 1996; Lehnert et al., 1996). For example, one characteristic feature of introns at positions 1046, 1199, 1210, 1389, and 1516 from Cladonia is a relatively short P5 region. This character is also found in homologous introns of other lichen-forming fungi, e.g. in
<table>
<thead>
<tr>
<th>Insertion position</th>
<th>114</th>
<th>287</th>
<th>323</th>
<th>392</th>
<th>516</th>
<th>531</th>
<th>789</th>
<th>943</th>
<th>956</th>
<th>1046</th>
<th>1052</th>
<th>1199</th>
<th>1210</th>
<th>1389</th>
<th>1506</th>
<th>1512</th>
<th>1516</th>
</tr>
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<tbody>
<tr>
<td>Lichen-forming fungi</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Other organisms</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>
Fig. 5.2. Putative secondary structure of a group I intron at position 1210 in the nuclear SSU rDNA of *Lecanora muralis*. Representation according to Cech et al. (1994).
DePriest and Been (1992) suggested that transacting factors are required for correct splicing because the Cladonia introns are missing in mature rRNA.

In addition to group I introns, an increasing number of relatively short insertions of less than 100 nucleotides in length has been described in lichen-forming and free-living fungi (Rogers et al., 1993; Untereiner et al., 1995; Grube et al., 1996). These short insertions are located at known intron positions, and some of them disrupt essential regions of mature rRNA. Rogers et al. (1993) showed, by RT–PCR, that short insertions are cut out from mature transcripts, although the splicing mechanisms remain unclear. Gargas et al. (1995b) used the term degenerate introns for short insertions and Grube et al. (1996) suggested that they may be the vestiges of initially complete introns, which lack the P3–P9 regions.

Phylogenetic relationships of various insertion site lineages of introns have been investigated for green algae by Bhattacharya et al. (1994, 1996), who showed an apparent monophyly of introns at positions 1506 and 1512, respectively. Generally, the introns at homologous positions have almost identical sequences in the conserved regions of the catalytic core. Increased sequence variation is, however, observed at peripheral regions. The sequence of the P2 region, considered as a spacer by Peyman (1994), and the P9 region are the most variable parts of the introns. Additionally, by analysing the known lichen introns, Bhattacharya et al. (1996) suggested that the closer relationships of introns at position 1199 and 287 and of introns at position 1210 and 1389 may be due to an ancient lateral transfer of an intron. This view is underlined by similarities of the 5′ flanking regions and the P10 pairing in each of the lineages.

We prepared a preliminary phylogenetic analysis of introns at position 1516, which are frequently found in Lecanorales (Fig. 5.3). Introns at this position from various representatives of Lecanorales can be aligned and parsimony analysis shows partial incongruency with the current classification of the lichens studied. The Cladonia introns appeared together with introns from Lecanora and Parmelia. This branch represented a sister group to introns from Physconia. According to the current concept of the Lecanorales, Cladonia would be a basal clade in this selection of taxa. The incongruency might be due to horizontal transfer of introns within the Lecanorales. This is also consistent with the theory of ‘late introns’, i.e. introns which are inserted secondarily into a particular position during rDNA evolution (see DePriest 1995). As introns in each of the genera group together it is probable that horizontal transfer of divergent introns is an ancient or rare event in Lecanorales. Conversely, there is evidence for a considerable rate of intron mobility within populations of a single species (see below).

It is still unclear whether introns are evenly distributed throughout the ribosomal repeat. It has repeatedly been observed that more than one fragment is amplified even from DNA of a single thallus (unpublished work). That these phenomena are due to the presence of lichenicolous fungi can be
excluded by microscopic examination. On the other hand it is possible that a lichen thallus is occasionally composed of more than one individual since fusion of different lichen thalli and mechanical hybridization can be observed occasionally in nature (see below). Alternatively, DePriest (1993a, b), Gargas et al. (1995b) and Crespo et al. (1997a) mentioned that more than one repeat type had been found even in single individuals of mycobionts. Gargas et al. (1995b) stated that the variable occurrence of insertions in the rDNA-repeat may produce anomalous PCR amplifications. We occasionally observe that even in those cases where a single intron-containing product was amplified, an intron-less product is obtained when a primer is used that spans the intron position, and which does not, therefore, amplify repeat units that contain the intron (unpublished results; see Gargas and DePriest, 1996). The reasons for these phenomena are unclear; however this is a challenging issue for future research. It is also important in this context to consider that the number of rDNA repeat units may change during vegetative growth (Pukkila and Skrzynia, 1993) and that meiotic recombination of rDNA is suppressed in certain fungi (Cassidy et al., 1984; Russell et al., 1988).

**Fig. 5.3.** Phylogenetic analysis of introns at position 1516 from various representatives of the Lecanorales. Strict consensus tree from three most parsimonious trees found using the branch and bound algorithm of the PAUP program (Swofford, 1991). The *Tetrahymena thermophila* intron (Tt.LSU) was used as an outgroup.
5.7 Genetic Variability of Populations

Lichen species may exhibit a high degree of morphological variation (Poelt, 1994). In many cases, this variation is apparently due to different ecological conditions. On the other hand, numerous examples are known where significantly different morphotypes of the same species grow side-by-side (Poelt, 1994). This indicates that certain phenotypic variation is also inherited as a genetic trait, which is also the case for chemical polymorphisms (Culberson et al., 1988). However, whereas there is now evidence that secondary compounds are produced by the mycobiont alone, it has not been estimated how much of the morphological variation may be attributed to different populations of photobionts within a lichen thallus. Independent genetic characters are therefore of great importance for lichen population studies.

Ribosomal DNA offers characters for direct investigation of mycobiont populations. Great variability of fungal rDNA within a lichen population was first demonstrated by DePriest and Been (1992). A single mat (about the size of a hand) of *C. chlorophaea* was composed of 13 different genotypes. A detailed analysis of these genotypes was presented by DePriest (1994) by using restriction site patterns and Southern hybridization. The sharing of restriction patterns suggests that some chemotypes may be polymorphisms of a single species in the *C. chlorophaea* complex. Significant size polymorphism of PCR products already show that this tremendous variation is due to the variable occurrence of insertions. Sequence analysis indicates that most insertions are group I introns in the coding regions of the rDNA.

DePriest (1995) used the information of the different genotypes to address questions of intron mobility in the *C. chlorophaea* complex. The phylogenetic hypothesis based on rDNA types suggests that it is most parsimonious to consider that both insertion and deletion of group I introns occur in rDNA of *C. chlorophaea*.

In different species of lichens, the heterogeneity of populations may vary. Whereas single mats of *C. chlorophaea* consist of many SSU rDNA genotypes, individual mats of *Cladina subtenus* are genetically uniform (Beard and DePriest, 1996). However, different mats of *Cladina* may be assigned to various genotypes. Three size classes of PCR products from the 3′ region of the SSU rDNA were detected among separate mats due to the presence of optional group I introns. Different PCR size-classes have also been described in a population study of *Parmelia sulcata* (Crespo et al., 1997b, 1998). In this study, PCR products from the ITS regions including the terminal region of the SSU and LSU rDNA were amplified. A significant increase in product size – of c. 200 nucleotides – was due to the presence of an intron at position 1516 in *P. sulcata*. Similar size variations were also observed in the related species *Parmelia saxatilis* (Fig. 5.4). In *Physconia*, the frequency of group I introns at position 1516 varied among different species (Cubero and Crespo, 1996). However, no correlation has yet been observed...
between the different intron frequencies and morphological variation, sexual mechanisms or ecological amplitude of species.

LaGreca (1996) presented a population study on the *Ramalina americana* complex, which included morphologically identical but chemically different races. Cladistic analysis of ITS sequence data suggested that the species complex consisted of two distinct groups. This grouping was also correlated with differences in chemical diversity and geographic distribution. The two groups were also supported by analyses of a second data set based on sequences of introns at position 1516 in the nuclear SSU rDNA.

An alternative approach to the study of lichen populations is possible using the random amplification of polymorphic DNA (RAPD) technique (Williams et al., 1990). Short primers are used to randomly amplify fragments which are interpreted as genetic markers. This technique may be applied to estimate variation over complete genomes. The only available RAPD study with lichens (Cubero et al., 1995) showed complex banding patterns with significant differences between populations from distant sites (Fig. 5.5). Further work is needed to evaluate the significance of RAPD analysis for population studies in lichens.

### 5.8 Miscellaneous Molecular Studies

Some miscellaneous studies address further issues of molecular lichenology. Glacier-covered lichens may serve as a source of ancient DNA. Gargas et al.
(1994) have isolated DNA from specimens of *Umbilicaria cylindrica* that were recently exposed after the glacier receded. Radiocarbon dating suggested that this material had been under the ice for 1300 years. Successful PCR amplification of DNA was possible from such apparently well preserved material. Therefore mummified lichens can be used to investigate sequence variation over historical time periods.

Armaleo and Clerc (1991) analysed different photomorphs of single lichen-forming fungi in the genera *Pseudocyphellaria* and *Sticta*. Fragments 2.2 kb in length were amplified from the 5‘ region of nuclear LSU rDNA. The products were then digested by restriction enzymes. Subsequent agarose gel analysis of the restriction fragments showed a ‘near identity’ of the different photomorphs. The similarity of the restriction patterns can be interpreted as molecular evidence that the corresponding photomorphs belong to the same fungal species.

PCR product length may also be used to detect distinct genetic individuals in lichen associations. Both partners of the lichen symbiosis may consist of more than one individual. This was suggested by indirect evidence from morphological and isozyme analyses (summarized by Fahselt, 1996) and mechanical hybridization between different initial thalli is a common explanation for such multisymbioses (see Fig. 5.10 in Honegger, 1996). Fahselt (1996) suggested that if intrathalline electrophoretic differences are generated by multiple symbionts, it is likely that they are related to the mycobiont, since

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**Fig. 5.5.** Random amplification of polymorphic DNA (RAPD) from *Parmelia saxatilis* (Cubero et al., 1995). Lanes 1 and 2, specimens from Antarctica (South Settlands, Livingston Island); lanes 3, 4 and 5, specimens from Spain (Sierra de Allón); lanes 6, 7 and 8, specimens from Spain (Ávila, Sierra de Gredos).
fungal biomass predominates in lichen thalli. Further evidence for this hypothesis is found by rDNA size polymorphisms within thalli of *P. sulcata*. Multiple amplification products were found with a frequency of 7% (n = 261 thalli; A. Crespo, unpublished work) in single thalli using fungal-specific primers (ITS1F and ITS4; Gardes and Bruns, 1993). PCR products of different length have been amplified from DNA isolations along a gradient through a thallus (Fig. 5.6). These data may indicate that more than one fungal rDNA genotype is present in a single thallus of *P. sulcata*.

### 5.9 Conclusions

PCR is a powerful technique for the investigation of the genetic variation in nuclear rRNA genes of lichen-forming fungi. The newly available sequence analyses contribute much to our understanding of lichen phylogeny. More representatives of the different orders of ascomycetes will be sequenced and this will clarify our views on the evolutionary origins of lichenization.

Investigations at lower taxonomic levels will improve our knowledge about evolution within and among lichen populations, species, genera, and families. It is now possible to link genetic information with that of morphological and chemical characters to study convergent evolution in different groups of lichen-forming fungi.

The frequency of insertions and introns is regarded as a useful character for population studies on lichens. The sequence diversity of mycobiont introns will also offer new information about the molecular evolution of ribozymes.

Future work will include investigations of various, yet poorly explored
genes. The ITS regions, LSU rDNAs and protein genes will be important additional sources of characters for phylogenetic studies. Beside further studies on ribosomal genes, a major challenge for the next years will be to study directly the genes which contribute to the tremendous chemical diversity of lichen compounds. Armaleo and Miao (1996), Miao and Davies (1998) and Miao et al. (1996) have already made advances in this direction.

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Applications of PCR for Studying the Biodiversity of Mycorrhizal Fungi

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6.1 Introduction

Mycorrhizal fungi are a heterogeneous group of about 6000 species belonging to the Zygo-, Asco- and Basidiomycotina. They are grouped into a number of different types depending on their morphological relationships with the host plants, which comprise about 240,000 plant species. Mycorrhizal fungi occupy different niches during their life cycle: they reside in the rhizosphere as spores, hyphae and propagules, they occupy the rhizoplane during their interaction with the root, and finally develop inside the root tissues during the symbiotic phase (Bianciotto and Bonfante, 1998). Despite the diversity of the taxa involved, mycorrhizal fungi share substantial features. They live in close association with the roots and accomplish their life cycle due to the establishment of symbiotic relationships. During the interaction, a bidirectional transfer of mineral nutrients and carbon occurs, ensuring a continuous flow of nutrients between the partners (Smith and Read, 1997). There is extensive literature on the molecular, cellular and physiological aspects of mycorrhizal fungi (Allen 1992; Gianinazzi and Schuepp, 1994; Bonfante and Perotto, 1995; Martin et al., 1995; Harrison, 1997). The positive effects of mycorrhizal fungi on plant nutrition, health and soil stability have valuable agrobiotechnological importance for low-input agriculture. However, previous knowledge of the biological diversity of mycorrhizal fungi in the rhizosphere is important to the potential exploitation of these fungi in agrobiotechnological systems. For example, the physiological traits and effectiveness of mycorrhizal fungi differ widely, depending on their taxonomic position and (at a lower rank) on the individual isolate. PCR-
based techniques have been used to provide molecular tools for the identification of both endo- and ectomycorrhizal fungi when their morphological characters are ambiguous or missing. They have also been used to examine relations between closely related species and populations of a single species, a level of resolution usually beyond the reach of classic morphological studies.

### 6.2 Molecular Tools to Study Biodiversity

According to Bruns and Gardes (1993), the ideal region of choice for PCR amplification should be: (i) present in all fungi of interest; (ii) easy to amplify; (iii) preferentially amplified from fungi, when plant and fungal DNAs are mixed; and (iv) variable enough to enable probes to be designed for several taxonomic hierarchies (species, genera, families). Genes coding for ribosomal RNA (rDNA) satisfy many of these criteria, and have been extensively analysed. Repeated copies of rDNA genes are present in the genome of both prokaryotes and eukaryotes. They are highly conserved and composed of different regions (Fig. 6.1). The ribosomal coding regions are the best conserved, the internal transcribed spacers (ITS) display a certain degree of variation, and the intergenic spacers (IGS) are the most variable regions. Amplification of these regions is normally followed by restriction fragment length polymorphism (RFLP) analysis of the PCR-generated fragments.

Other PCR-based techniques have also been used to study the biodiversity of mycorrhizal fungi. Random amplification of polymorphic DNA (RAPD) is based on the use of short oligonucleotides of random sequence. This technique has proved to be a powerful tool in revealing genetic differences between closely related organisms, and has been extensively used in ecological studies to investigate the biodiversity of natural populations (Hadrys et al., 1992; Foster et al., 1993).

PCR amplification of regions containing microsatellites has been less commonly used in mycorrhizal research. Microsatellites are a special class of tandem repeats that involve a base motif of 1–10 bp repeated up to 100 times (Tautz, 1993) and dispersed over many genomic loci. They are present in eukaryotic genomes and have been used in individual identification, parentage testing, population genetic studies, genome mapping and in the screening of a wide range of species and populations.

![Fig. 6.1. Scheme showing a rDNA repeat. The positions corresponding to the annealing sites for several primers are shown by arrows.](image-url)
of genomic libraries. Their exceptionally high mutation rate makes microsatellites very informative for determining relationships between closely related species (Bowcock et al., 1994; Goldstein and Clark, 1995).

The aim of this chapter is to review the recent literature on PCR techniques applied to mycorrhizal symbionts, and to discuss their implications in soil microbial ecology.

6.3 **PCR-based Methods to Investigate Population Dynamics of Ectomycorrhizal Fungi**

More than 5000 species of ectomycorrhizal (ECM) fungi are associated with the secondary roots of Gymnosperms and Angiosperms (Molina et al., 1992). One of the major objectives of current research is to understand the structure and dynamics of these fungal communities (Dahlberg and Stenlid, 1995). ECM fungi in fact display a great diversity even in small monoculture forests, which are typically occupied by 20–35 species (Bruns, 1995). Prior to the introduction of PCR methods, community studies focused on the presence of sporocarps with the assumption that their production reflected the abundance of symbiotic mycelia (Dahlberg and Stenlid, 1995). The presence of ECM fungi can also be evaluated through their morphological identification on mycorrhizal root apices; however, caution should be used because the extent of intraspecific morphological variations between taxa and in the same taxon on different hosts or in different environments is not well known (Egger, 1995). The PCR methods have advantages when applied to any stage in the life cycle of a mycorrhizal fungus including fruiting bodies, mycorrhizas, extraradical mycelia and isolated mycelia growing *in vitro*.

The nuclear and mitochondrial genes encoding structural rRNAs have been used to identify ECM fungi (Gardes et al., 1991). Bruns and Gardes (1993) designed probes by the alignment of partial sequences of the mitochondrial large subunit rRNA gene (mt-LSU), which were specific for several taxa within the suilloid group of the *Boleta*. The probes were targeted at *Suillus*, *Rhizopogon*, and *Gomphidius*, and their specificity was determined by testing their ability to hybridize to PCR amplified fragments from 84 basidiomycete species. The probes were mostly useful in the identification of suilloid fungi at the generic level.

The original universal primers designed by White et al. (1990) to amplify the nuclear ITS region have been followed by primers for the amplification of specific taxa. Gardes and Bruns (1993) designed two taxon selective primers intended to be specific for fungi (ITS1-F) and Basidiomycetes (ITS4-B) respectively, though they can often amplify plant DNA. One limitation in the use of the ITS region is that the level of intraspecific variability is not uniform for all species. On the other hand, the ITS region is, in general, sufficiently variable to allow the clear discrimination between distantly related species and related genera. The increasing number of available
sequences of ribosomal genes has also allowed the construction of phylogenetic trees. For example Kretzer et al. (1996) have revised the genus *Suillus* by analysing 38 isolates belonging to many species. On this basis, they proposed a phylogenetic tree and suggested interesting relationships between the EMF and their host plants.

The development of PCR-based protocols has initiated many programmes for the investigation of the extent of biological diversity in field conditions. In a study carried out in a Norwegian spruce forest in Sweden, Erland (1995) used the universal primers ITS1 and ITS4 to amplify the ITS region from DNA extracted from mycorrhizal tips. Five major RFLP patterns were found from the amplified products and one was identified as typical of *Tylospora fibrillosa*. This fungus was present in at least 21% of the mycorrhizal roots tested, and was regarded as one of the most abundant mycorrhizal fungi. Similarly, Karen et al. (1996) compared RFLP patterns of ITS products amplified from mycorrhizal roots with those obtained from fruiting bodies of reference species. In this investigation, the fruiting body inventory indicated that the clear-cut planted forest had the lowest diversity and species richness, despite the fact that it showed the highest number of fruiting bodies. A forest of 150- to 200-year-old trees produced less fruiting bodies, but examination of mycorrhizal tips revealed several species which did not form epigeous fruiting bodies. In conclusion, the results suggested that forestry management affects fruiting body diversity more than mycorrhizal diversity (Karen et al., 1996). Another interesting study of natural ECM populations was performed by Gardes and Bruns (1996). They examined the species diversity of a community in a forest of *Pinus muricata* in order to determine the above- and below-ground correspondence, in terms of species composition, spatial frequency and abundance. ITS-RFLP, taxon-specific oligonucleotide probes, and sequence analysis were applied to fruiting bodies and mycorrhizal tips sampled over a 4-year period. Over 45 fungal species were identified and grouped according to three patterns: (i) some, such as *Russula xerampelina*, were well represented both above and below ground; (ii) some, such as *Suillus pungens*, were mostly found as epigeous fruiting bodies; (iii) some, such as *Russula amoenolens* were not represented in the above-ground species. These results demonstrated that ECM fungal species change their resource allocation towards the production of either fruiting bodies or ectomycorrhizae in the same environment. Moreover, it is clear that there is no correspondence between above- and below-ground fungal structures (Gardes and Bruns, 1996).

The fate of an introduced ECM strain has been followed in a long-standing study by the group of Martin and Le Tacon, who monitored the presence of *Laccaria bicolor* strain S238N, an isolate known to improve the growth of Douglas fir and Norwegian spruce in nurseries. To evaluate the competitiveness and persistence of the isolate after transplantation, the mycotrophic status of the forest trees was examined morphologically and determined by ribotyping between 1 and 10 years after planting out. *Laccaria*
was present in 90% of the mycorrhizal tips after 1 year, but in only 3% after 4 years, since it had been widely replaced by local strains. However, in other forest sites, *Laccaria* persisted for at least 10 years. These results showed that the destiny of an inoculated strain depends on the host plant, the presence of competitive local strains, and/or the climatic conditions (Henrion et al., 1994b; Di Battista et al., 1996; Selosse et al., 1996).

In southern Europe, truffles have been the subject of intense application studies since the 1970s due to the outstanding commercial value of some species (Chevalier, 1994). Identification of truffles during their symbiotic phase is one of the major topics in this kind of research. The fruiting bodies are usually identified on the basis of the structure of the peridium and gleba, size and shape of their spores and asci, and their wall ornamentation. However, these features are lost when the hyphae of the mantle and Hartig net develop on the plant root. Many fruiting bodies have been easily identified with primers which amplify the ITS regions (Henrion et al., 1994a; Lanfranco et al., 1995a; Paolocci et al., 1995), whereas many difficulties remain when mycorrhizal roots are considered. ITS primers often amplify plant DNA and, therefore, can give rise to complex patterns (Mello et al., 1996).

Strategies can be developed to allow the exclusive amplification of fungal DNA from species of interest. For example, sequences of ITS fragments amplified from fruiting bodies have been analysed in order to design specific primers which permit the exclusive detection of the fungus of interest (Mello et al., 1997) (Fig. 6.2). These specific sequences generally work at the species level, but only when the degree of intraspecific variation of the ITS region is low, as has already been demonstrated for some truffle species (Guillemaud et al., 1996).

PCR amplification of single copy genes is an alternative approach. For example, Kreuzinger et al. (1996) have successfully used this method on a 1.2 kb fragment of the gene encoding glyceraldehyde-3-phosphate dehydrogenase. The amplified fragments from *Boletus, Lactarius*, and *Amanita* were distinguished by RFLP analysis and Southern blot hybridization, while fluorescent DNA probes could easily be used in slot-blot experiments. This approach is an original way for quickly developing inexpensive probes to detect ECM fungi of ecological and economic value.

### 6.4 Arbuscular Mycorrhizal Fungi: from the Development of Specific Probes to Ecological and Genetic Analysis

Arbuscular mycorrhizal (AM) fungi are obligate endosymbionts that colonize the roots of almost 80% of land plants. They belong to the Order *Glomales* (Morton and Benny, 1990), and consist of least 150 species, usually identified by their spore morphology (Walker, 1992). Simon et al. (1992, 1993a, b) were the first to apply PCR techniques to AM fungi in a study of
the nuclear gene encoding the small subunit rRNA. From the complete 18S sequence of two AM species (*Glomus intraradices* and *Gigaspora margarita*), they designed a primer (VANS1) that only amplified *Glomales* species, when used in combination with universal primers. This combination has successfully amplified the fungal DNA in mycorrhizal roots colonized by *Glomus vesiculiferum* (Simon *et al.*, 1992). When more 18S sequences from other AM species became available, family specific primers were also designed and used on mycorrhizal roots. The amplified products were then subjected to single-strand conformational polymorphism (SSCP) analysis to detect sequence differences (Simon *et al.*, 1993b).

The sequence data of the small ribosomal subunit from 12 isolates of

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**Fig. 6.2.** Design of specific primers for *Tuber* species. (A) Amplification products with ITS1 and ITS4 primers on DNA extracted from the fruiting bodies of ten *Tuber* species. Lanes: 1, pBR 322 digested with *Hind*; 2, *T. magnatum*; 3, *T. borchii*; 4, *T. maculatum*; 5, *T. melanosporum*; 6, *T. macrosporum*; 7, *T. rufum*; 8, *T. aestivum*; 9, *T. brumale*; 10, *T. excavatum*; 11, *T. ferrugineum*; 12, no DNA. (B) On the basis of the ITS sequence, specific primers were designed for *T. borchii* and used to amplify a single band of 432 bp from fruiting bodies (lane 2), ectomycorrhizae (lane 3) and mycelium (lane 4). No amplification is obtained from other truffle species (lanes 5–11).
glomalean fungi have been used to map the phylogenetic relationships between AM fungi (Simon, 1996). The results confirmed the validity of the taxonomic grouping and allowed Simon et al. (1993a) to construct a molecular clock, by estimating the divergence times between glomalean families and genera.

The development of molecular markers for the identification of individual spores has been an important step for investigating species diversity of AM fungi in the rhizosphere of natural communities (Clapp et al., 1995; Sanders et al., 1995, 1996; Dodd et al., 1996). Sanders et al. (1995) developed a protocol for PCR amplification with the universal primers ITS1 and ITS4, followed by RFLP to characterize the ITS region from single AM fungus spores. The ITS region, which is approximately 600 bp long, showed clear differences between species only after RFLP; the restriction patterns were reproducible for individual spores of a given species. However, when the technique was applied to spores from a Swiss grassland, the results were much more complex: ten individual spores belonging to the same morphological Glomus group produced ten different patterns, suggesting a high genetic diversity between individual spores.

Clapp et al. (1995) used PCR and genus-specific primers designed from 18S rDNA to investigate the fungal composition of mycorrhizal roots collected in the field. They introduced the selective enrichment of amplified DNA (SEAD) technique, based on the principle of subtractive hybridization, to remove interfering plant-derived DNA. Three genera of AM fungi, Acaulospora, Scutellospora and Glomus, were detected in bluebell roots. While the presence of the first two genera was expected on the basis of the spores found in the soil around the roots, Glomus spores were very rare in the rhizosphere, suggesting that the presence of an endophytic fungus does not always correlate with the presence of propagules.

Alternative PCR-based strategies have been devised to identify AM fungi during the sporal and symbiotic phases. Short arbitrary oligonucleotides were used as primers for the amplification of DNA extracted from spores of AM fungi in RAPD experiments (Wyss and Bonfante, 1993; Lanfranco et al., 1997). RAPD analysis is a very sensitive tool for the detection of genetic differences between individuals. A non-polymorphic band was identified as a marker for eight isolates of Glomus mosseae. The fragment (about 650 bp long) was cloned and sequenced, and a pair of specific primers were designed (Lanfranco et al., 1995b). These specifically amplified not only the DNA from G. mosseae spores, but also from roots of pea, clover, leek and onion plants when they were colonized by G. mosseae isolates. In addition, they allowed G. mosseae to be distinguished from the closely related species G. coronatum: interestingly, the two species were not distinguishable by ITS amplification techniques (Fig. 6.3) (Dodd et al., 1996).

Highly repeated sequences are also widespread in the fungal genome. Their presence has been demonstrated in AM fungi by two approaches:
Longato and Bonfante (1997) have used primers designed on microsatellite sequences, such as (CT)_8, (GACA)_4, (TGTC)_4, to obtain species-specific amplification profiles. This approach has proved to be a reliable and technically simple method for assaying genetic variability. Zézé et al. (1996) have isolated a highly repeated sequence from a partial genomic library of AM fungi. A single band of about 500–560 bp was obtained when the following isolates were investigated: 1, *Glomus coronatum*; 2, *G. mosseae*; 3, *G. versiforme*; 4, *G. flavusporum*; 5, *Gigaspora margarita*. Different patterns were obtained after RFLP analysis with *Hin*II. (B) RAPD analysis performed using the primers OPA-7, OPA-11 and OPA-18 (Operon Technologies, USA) on: 1, *Glomus coronatum*; 2, *G. mosseae*; 3, *Gigaspora margarita*. Highly polymorphic patterns, which allow a clear separation between *G. coronatum* and *G. mosseae* were obtained. M, λ DNA digested with *Hind*III and *Eco*RI (from Dodd et al., 1996 by permission of New Phytologist, Cambridge University Press).

**Fig. 6.3.** Genome analysis on AM fungi. (A) Amplification products with ITS1 and ITS4 primers on DNA extracted from AM fungi. A single band of about 500–560 bp was obtained when the following isolates were investigated: 1, *Glomus coronatum*; 2, *G. mosseae*; 3, *G. versiforme*; 4, *G. flavusporum*; 5, *Gigaspora margarita*. Different patterns were obtained after RFLP analysis with *Hin*II. (B) RAPD analysis performed using the primers OPA-7, OPA-11 and OPA-18 (Operon Technologies, USA) on: 1, *Glomus coronatum*; 2, *G. mosseae*; 3, *Gigaspora margarita*. Highly polymorphic patterns, which allow a clear separation between *G. coronatum* and *G. mosseae* were obtained. M, λ DNA digested with *Hind*III and *Eco*RI (from Dodd et al., 1996 by permission of New Phytologist, Cambridge University Press).
Scutellospora castanea. This DNA fragment has been shown to be specific for this species by Southern blot hybridization, and by PCR assays with oligonucleotides derived from the sequence.

Molecular markers targeted to the ribosomal genes have provided new information. Sanders et al. (1995) have shown that a single spore of *G. mosseae* contains at least two different ITS sequences. Moreover, a third ITS variant has been found by Franken and Gianinazzi-Pearson (1996) by sequencing a ribosomal clone from a genomic phage library of *G. mosseae*. Lloyd-MacGillp et al. (1996) have made it clear that ITS heterogeneity is normal within *Glomus* spores. They obtained two sequences from single spores of three *G. mosseae* isolates and one isolate of *G. dimorphicum*, and three sequences from a single *G. coronatum* spore. Interestingly, the sequence divergence between isolates from distant continents is scarcely greater than that within single spores. The authors concluded that the sequence variants have evolved over a long time-scale relative to the rate at which these fungi spread across the world.

Some recent data obtained from the *Gigasporaceae* have shown a similar situation for this family. Three ITS sequences have been identified by cloning the amplified ITS fragment from *Gigaspora margarita* (L. Lanfranco, unpublished work), although it should be noted that these were obtained from a multispore DNA preparation. More compelling is the finding of different 18S sequences in a single spore of *Scutellospora* collected in an oak woodland in the north of England (J.P. Clapp, unpublished work, cited by Sanders et al., 1996). These results show a high level of genetic variability in genes belonging to the same genome (Sanders et al., 1996) and in organisms thought to be asexual. The many questions they raise have been discussed in detail by Sanders et al. (1996) who suggest that the multinucleate spores and hyphae of Glomales may be the products of heterokaryotic processes, and the variability of ITS sequence may be related to asexual reproduction.

In conclusion, PCR-based molecular markers for the identification of AM fungi allow both the investigation of their biodiversity in the rhizosphere and within the roots, and the elaboration of a phylogenetic framework. These methods have also opened the way to an understanding of AM fungal genetics.

### 6.5 PCR-based Methods to Analyse the Complexity of Ericoid Mycorrhizal Fungal Populations

Ericaceous plants associate with soil fungi to form a distinctive type of mycorrhiza, termed ericoid mycorrhiza (Perotto et al., 1995). The specific features of this symbiosis make ericoid mycorrhizal plants successful in colonizing low-mineral, acidic organic soils high in toxic metal ions, where a crucial role in plant nutrition has been ascribed to the saprotrophic capabilities of the mycorrhizal endophyte. The endophytes isolated so far
belong to the ascomycetes, although basidiomycetes have been observed by electron microscopy inside naturally colonized roots (Bonfante, 1980). Few fungal species have been identified as partners of this mycorrhiza: *Hymenoscyphus ericae* was the first (Read, 1974), and several *Oidiodendron* species have also been demonstrated to be mycorrhizal in several parts of Europe and North America (Couture et al., 1983; Dalpé, 1986; Douglas et al., 1989; Perotto et al., 1996a). However, the taxonomic position of most isolates is unknown, because they do not form identifiable reproductive structures. Isolates of *H. ericae* are usually hard to identify, as they often grow as sterile mycelia in pure culture.

Investigation of the genetic diversity of ericoid fungi has greatly benefited from PCR techniques as a means of overcoming the difficulties of morphological identification. By coupling molecular methods and traditional taxonomy, new tools have been provided for the study of ericoid mycorrhizal fungi. PCR–RFLP analysis of different regions of the ribosomal genes has been used to investigate the identity and diversity of ericoid fungi. Examination of the small subunit of the ribosomal genes of the hyphomycete *Scytalidium vaccinii* revealed its close taxonomic relationship with *H. ericae* (Egger and Siegler, 1993), while RFLP analysis of the ITS region amplified from mycelia colonizing *Calluna vulgaris* roots has demonstrated that the root of a single plant harbours several populations of mycorrhizal and non-mycorrhizal fungi (Perotto et al., 1996a). Investigation of mycelia isolated from *Gaultheria shallon* roots (Monreal et al., 1996) has also revealed the contemporary presence of fungi with different ITS restriction patterns.

RAPD analysis has enabled a higher resolving power to be used in the investigation of the genetic polymorphism of isolates sharing the same ITS-RFLP pattern (Perotto et al., 1996a). Results of PCR amplification with about ten random primers on about 80 mycorrhizal mycelia isolated from *C. vulgaris* growing in five neighbouring sites have shown a high polymorphism in *Oidiodendron maius* isolates, even those derived from the same plant, and a lower variability within populations of sterile mycelia (Fig. 6.4). These data indicate that the root system of a single plant of *C. vulgaris* is a complex mosaic where several populations of mycorrhizal fungi coexist, each represented by a variable number of genetically distinct individuals (Perotto et al., 1996a). The ecological significance of this promiscuous association is not clear. However, several ericoid isolates produce specific isoforms of extracellular enzymes useful for nutrition *in vitro* (Perotto et al., 1997). Thus, an association with several fungi may be a way of broadening the metabolic capabilities of mycorrhizal roots in the exploitation of difficult substrates.

Analysis of ribosomal genes in ericoid fungi has revealed an unusual feature in their organization in many isolates. Amplification using universal primers designed to both the 18S and the 28S subunits has yielded DNA fragments which were often much larger in size than expected (Egger et al., 1995; Perotto et al., 1996b). Sequencing of these fragments has revealed that this discrepancy was due to the insertion of group I introns.
Group I introns have four conserved regions involved in the formation of secondary structures that are important for splicing. These elements are quite rare in the nuclear ribosomal genes of eukaryotes. They occur sporadically in a few fungal species and in algae, but their role has not been elucidated (Johansen et al., 1996). Interestingly, they are abundant in the polyphyletic group of lichen-forming fungi (Gargas et al., 1995).

The distribution and sequence of group I introns are highly variable in ericoid fungi. One intron element inserted in the region towards the 3′ end of the small ribosomal subunit in H. ericae is well characterized (Egger et al., 1995) and a number of additional insertions have been found in this and other ericoid fungal isolates (Perotto, unpublished work), suggesting that this may be a common feature. Although their role is not known, introns certainly increase the genetic diversity of ericoid fungi.

In conclusion, PCR-based techniques are a powerful tool for the study of ericoid fungi: the identification of isolates through specific fingerprinting methods is quickly overcoming the lack of morphological characters often encountered with these fungi.

6.6 The Interactions between Mycorrhizal Fungi and Endosymbiotic Bacteria

External hyphae of mycorrhizal fungi encounter many other soil microorganisms in the rhizosphere, and a network of interactions is created (Azcon-Aguilar and Barea, 1992). PCR has also been of great assistance in this field, particularly in the investigation of the interactions between
mycorrhizal fungi and bacteria. The surface of both ECM and AM fungi can be colonized by soil bacteria that use the hyphae as a specific substrate (Bianciotto et al., 1996a; Schelkle et al., 1996). Furthermore, the cytoplasm of AM fungi harbours structures called bacteria-like organisms (BLO). Identification of these bacterial endosymbionts has been hampered because they cannot be grown on cell-free media. Amplification and partial sequencing of the 16S rDNA region of endosymbiotic bacteria, followed by database searches for sequence similarity and phylogenetic analyses, have now unambiguously placed the symbiont of Gigaspora margarita among the pseudomonads of rRNA group II (Bianciotto et al., 1996b). The endosymbiont was identified as a member of the genus Burkholderia. The sequence of the gene coding for the small subunit rRNA has been used to design two primers (BLOf and BLOr) specific for the Burkholderia endosymbiont of Gi. margarita. These primers were tested for their specificity on a number of free-living bacteria, and then used on resting spores, external mycelium and clover roots mycorrhized with Gi. margarita in order to follow the fate of BLOs during the fungal life cycle. Spores of different origins belonging to Glomaceae, Gigasporaceae and Acaulosporaceae, were analysed by confocal microscopy using a fluorescent dye specific for the visualization of bacteria to determine whether other AM fungi possess bacterial endosymbionts. In addition, PCR amplifications with eubacterial primers (704f/1495r) and with BLOf/BLOr were performed on DNA preparations from spores of different isolates. All spores, with one exception, contained intracellular bacteria, although their number and shape greatly differed among the different species. In addition, the BLOf/BLOr primers were found to amplify only a DNA fragment from Gigasporaceae spores (Bianciotto et al., 1996c).

In conclusion, these experiments suggest that intracellular bacteria are not a sporadic phenomenon but may be a general feature of AM spores. Their presence raises many questions about their role in mycorrhizal functions and about evolutionary processes and it has been suggested that they may be organelle precursors (Holzman, 1996).

6.7 Areas that Need Further Developments

Despite the huge progress of recent years, many problems remain unresolved; most of these are of a technical nature. For example, it is important to improve the level of amplification for samples collected in the field; at present this level is unsatisfactory (Harris, 1996). This is probably caused by the presence of inhibitory substances.

Another important point that has rarely been tackled is the quantification of DNA in the samples. Simon et al. (1992) were the first to propose a protocol for estimation of the amount of AM fungi living inside the plant roots by amplifying the sample with different concentrations of an internal standard.
Edwards et al. have developed an alternative protocol and tested the possibility of evaluating the effect of rhizosphere bacteria on the development of AM fungi (S.G. Edwards, A.H. Fitter and J.P.W. Young, 1997, personal communication). A more precise scenario of the competitive colonization of roots by mycorrhizal fungi will only be available when it is possible to quantify the presence of one fungal genome with respect to another.

6.8 Conclusions

The development of PCR-based techniques has allowed us to better define the presence and role of mycorrhizal fungi in the rhizosphere, which is a complex niche where microorganisms develop and interact with each other. Because of PCR-based techniques (Fig. 6.5), many crucial questions about phylogenesis, identification and polymorphisms of mycorrhizal fungi have begun to be answered. For example, the extent of diversity in natural populations has been probed and shown to be greater than previously thought. Several symbiotic fungi are often present, not only in the same environment, but on the same root irrespective of its size. The thin roots of Ericales are a good illustration of this phenomenon. The dynamics of the resident fungal population have also been directly evaluated, as well as the fate of introduced strains selected for their physiological properties. It is clear that the ability of these strains to survive depends on the environment, as well as on their interactions with other soil microorganisms.

Fig. 6.5. A flow chart showing the most important PCR-based strategies that have been used to investigate the genome of mycorrhizal fungi.
The genetics of mycorrhizal fungi is still in its infancy, particularly in the case of AM fungi, which are intractable organisms due to their obligate biotrophic nature. PCR-based techniques have allowed us to investigate these organisms inside the roots, to elaborate a phylogenetic framework and to give an insight into their genetics for the first time. Molecular tools have revealed an unexpected level of variability in the ribosomal genes in individual isolates. The discovery of several introns in the ribosomal genes of ericoid mycorrhizal fungi suggests similar conclusions, as such features are also a source of genetic variability.

Further technical developments are still needed to solve specific problems in mycorrhizal research. However, despite these current limitations PCR-based techniques have so far provided an invaluable contribution to the definition of the mycorrhizal fungal dimension in the rhizosphere.

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PCR Applications in Fungal Phylogeny

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7.1 Introduction

Historically, the phylogeny of fungi has been inferred from various phenotypic characters, e.g. morphological, physiological and developmental characters, and/or chemical components such as secondary metabolites. The recent development of molecular techniques has enabled the derivation of fungal phylogenies based on the analyses of proteins or nucleic acids, e.g. isozyme analysis, DNA–DNA hybridization, electrophoretic karyotyping, restriction fragment length polymorphism (RFLP), and DNA sequencing. However, these molecular techniques have been difficult to apply to obligate parasites from which only small amounts of fungal materials are available, or to rare taxa which are available only as herbarium specimens. The polymerase chain reaction (PCR) developed in the mid 1980s (Saiki et al., 1985; Mullis et al., 1986; Mullis and Faloona, 1987) made possible the amplification of particular nucleotide sequences from very small amounts of starting materials. In addition, relatively impure DNA extracts can be used for PCR. PCR-based techniques have enabled the construction of molecular phylogenies for obligate parasites and rare taxa, and as PCR techniques have led to simplified molecular procedures, they have also increased the availability of phylogenetic studies for many fungi. This chapter reviews some of the recent advances that have been made through the application of PCR methods to phylogenetic studies of fungi.
7.2 Sequence Analysis from Small Samples

7.2.1 DNA extraction

A number of methods have been developed for the rapid isolation of fungal DNA (Biel and Parrish, 1986; Zolan and Pukkila, 1986; Taylor and Natvig, 1987; Lee et al., 1988; Möller et al., 1992). Most of these are applicable to molecular phylogenetic analysis when large amounts of fungal materials can be obtained. However, a small amount of starting material is required for PCR-based sequence analysis, and sufficient single-copy genomic sequences can be routinely amplified from 1 µg of total DNA (Saiki et al., 1988). Lee and Taylor (1990) simplified an earlier method for total DNA isolation and used it for the amplification of multicopy genes from small quantities of fungal materials (Lee et al., 1988). They reported that rDNA sequences could be successfully amplified from a single spore of Neurospora tetrasperma. In molecular phylogenetic analysis, sequence data are often required from herbarium collections, especially when they concern rare taxa. Bruns et al. (1990) applied Lee and Taylor’s method to 35 collections of dried fungal materials stored under a variety of conditions for 1–50 years. They successfully amplified the mitochondrial large subunit rRNA gene from all of the specimens, and 16 specimens were subsequently sequenced.

Walsh et al. (1991) developed a method utilizing a chelating resin for extracting DNA from forensic-type samples for PCR. The method was simple, rapid, did not include organic solvents and did not require multiple tube transfers. This method has proved applicable to the extraction of DNA from small amounts of fungal materials, and Hirata and Takamatsu (1996) have used the method to obtain the rDNA internal transcribed spacer (ITS) regions from several obligately parasitic powdery mildew fungi.

7.2.2 PCR amplification

A variety of oligonucleotide primers have been designed for the amplification of rDNA sequences from a wide range of fungal materials (e.g. White et al., 1990). Amplification using these primer sets, followed by direct sequencing has considerably shortened the time required to perform phylogenetic studies.

In addition to universal primers, many kinds of primer sets for the amplification of rDNA have been developed for particular groups of fungi; two examples of this are lichenized fungi (Gargas and Taylor, 1992) and slime moulds (Rusk et al., 1995).

In most cases simple thermal cycling parameters have proved sufficient to amplify rDNA sequences; however, two sequential reactions using nested primers can improve the efficiency of amplification from small quantities of DNA (Hirata and Takamatsu, 1996).
7.2.3 Sequence analysis

Two methods are in general use for sequencing PCR-amplified DNA fragments. One procedure involves cloning. Although cloning procedures have become more straightforward, considerable effort is still required especially when obtaining sequence data from large numbers of individuals. Another disadvantage of the cloning methods is that mutations can be introduced by the DNA polymerase during synthesis. Taq DNA polymerase lacks editing functions and incorporates an incorrect nucleotide at a rate of $2 \times 10^{-4}$ nucleotides per PCR cycle. This rate of misincorporation translates into an overall error frequency of one per 400 bp after 30 cycles of amplification (Saiki et al., 1988). However, conditions can be optimized to reduce this error frequency to less than one substitution in 15,000 bp (Gelfand and White, 1990). As a result a consensus sequence, based on several cloned PCR products, has to be determined for each sample in order to distinguish mutations present in the original sequence from any random misincorporation of nucleotides introduced by the polymerase during PCR.

The second procedure involves direct sequencing of PCR product without cloning. This generates a consensus sequence at each nucleotide and minimizes possible errors from misincorporation. Since the procedure does not involve the cloning of the PCR product, the time required for comparative sequencing studies is significantly reduced. As a result, direct sequencing is being widely used for phylogenetic studies based on DNA sequences (Lott et al., 1993; Morales et al., 1993; Zambino and Szabo, 1993; Saenz et al., 1994; Nishida and Sugiyama, 1994).

7.2.4 Construction of phylogenetic trees

Many computer packages have been developed for phylogenetic studies, and six of the more widely used are described here.

**CLUSTAL W**

The program **CLUSTAL W** (Thompson et al., 1994) has a multiple sequence alignment function that works well with large numbers of either nucleic acid or protein sequences. The program also constructs phylogenetic trees by the neighbour-joining method of Saitou and Nei (1980), but does not draw the tree. Confidence intervals on the tree are calculated using a bootstrap procedure similar to that described by Felsenstein (1985).

**MACCLADE**

**MACCLADE** (Maddison and Maddison, 1992) is a program for analysing character evolution. It has a graphical spreadsheet editor for entering and editing data matrices, a tree window for viewing and manipulating trees, charting facilities, and other features. Although **MACCLADE** does not have a sophisticated search algorithm to find the best tree, it can be complemented by **PAUP** (see below) as the two packages are compatible.
MEGA
MEGA (molecular evolutionary genetics analysis; Kumar et al., 1993) is a phylogeny inference package that utilizes various distance methods and parsimony. Various other statistics are included for the analysis of DNA, RNA and protein sequence data.

PAUP
PAUP version 3 (Swofford, 1993) is probably the most sophisticated program for inferring phylogenies using parsimony analysis, with many options and good compatibility with MacClade. Version 3 itself is currently not available, but the new program, PAUP version 4, will be released in the summer of 1998. Version 4 will include parsimony, distance matrix, invariants, and maximum likelihood methods.

PHYLIP
PHYLIP (Felsenstein, 1989) includes programs for parsimony, distance matrix, and maximum likelihood methods for a variety of data types, including molecular sequences (DNA and protein), gene frequencies, continuous characters, discrete (0, 1) characters and restriction sites. It also includes programs for plotting phylogenies and consensus trees.

PUZZLE
PUZZLE is a package that uses maximum likelihood to reconstruct phylogenetic trees from sequence data. It implements a fast tree search algorithm (quartet puzzling; Strimmer and von Haeseler, 1996) that allows analysis of large data sets and automatically assigns estimations of support to each internal branch. Rate heterogeneity is incorporated in all models of substitution. All model parameters including rate heterogeneity can be estimated from the data by maximum likelihoods. PUZZLE also computes pairwise maximum likelihood distances as well as branch lengths for user specified trees, and offers a novel method, likelihood mapping, to investigate the support of internal branches without computing an overall tree.

7.3 Methods for Phylogenetic Analysis

7.3.1 DNA sequence analysis
DNA sequence analysis has become the most useful method for inferring phylogenetic relationships between organisms. Bruns et al. (1990) described the benefits of using DNA sequences for phylogenetic analysis as being ‘the large number of characters compared can substantially increase the resolving power’. One can also observe the mode of sequence variation, that is, whether a change is a transversion or transition, silent or selected, and one can measure the degree of nucleotide bias; results from different laboratories can be
compared directly, and the publication of sequences and their deposition in electronic databases (GenBank, EMBL and DDBJ) allows the confirmation of results and their application to other taxa without the need to obtain strains or clones, or to repeat experiments. Some DNA regions which have proved useful for sequence analysis for phylogenetic studies are described below.

**Nuclear and mitochondrial rDNA**

The nuclear rRNA genes (rDNA) of eukaryotes are arranged in tandemly repeated clusters with each cluster containing the genes for the small subunit (18S), 5.8S, and large subunit (25–28S) rRNA (Gerbi, 1985). These genes show little sequence divergence between closely related species and are useful for phylogenetic studies among distantly related organisms (Jorgensen and Cluster, 1988; Bruns et al., 1991; Hillis and Dixon, 1991; Illingworth et al., 1991; Taylor et al., 1993). Studies of the secondary structure of the rRNA have shown that eukaryotic large subunit rRNAs are composed of a conserved ‘core’ that has a similar secondary structure to prokaryotic rRNAs, and interspersed ‘divergent domains’ that appear to have no prokaryotic homologue (Michot et al., 1984; Hillis and Davis, 1987; Michot and Bachellerie, 1987). DNA sequences of the divergent domains of the nuclear large subunit rDNA are useful for determining phylogenies of relatively closely related organisms. Within each repeat, the conserved regions are separated by two internal transcribed spacers (ITS), which show higher rates of divergence (Jorgensen and Cluster, 1988; Ritland et al., 1993; Schlötterer et al., 1994). A third spacer, the large intergenic spacer (IGS), is found between the 3′ end of the 28S and the 5′ end of the 18S genes. These spacer regions evolve faster than the subunit genes and can be useful for studying closely related organisms, such as among species within a genus or among populations (Jorgensen and Cluster, 1988; Bruns et al., 1991; Ritland et al., 1993; O’Donnell, 1992; Hsiao et al., 1994, 1995; Yan et al., 1995; Kusaba and Tsuge, 1994, 1995). The mitochondrial rRNA genes evolve approximately 16 times faster than the nuclear rDNA (Bruns and Szaro, 1992), and so are useful for phylogenetic studies at an intermediate taxonomic level (Bruns et al., 1991; Simon et al., 1994).

**Protein genes**

Protein coding genes have some advantages over rRNA genes and spacers in that the alignment of the sequence is less problematic (Bruns et al., 1991). Protein sequences also lend themselves to differential weighting of bases by codon position, and third position sites can provide a relatively good estimate of the neutral substitution rate. Elongation factor-1a protein genes (Hasegawa et al., 1993; Baldauf and Palmer, 1993; Nordnes et al., 1994), actin genes (Baldauf and Palmer, 1993; Fletcher et al., 1994), β-tubulin genes (Baldauf and Palmer, 1993; Edlind et al., 1996), glyceraldehyde-3-phosphate dehydrogenase genes (Martin et al., 1993), and nitrate reductase genes (Zhou and Kleinhofs, 1996) have been used for phylogenetic analyses of eukaryotes.
including fungi. Actin genes (Cox et al., 1995; Wery et al., 1996), chitin synthase genes (Mehmann et al., 1994; Karuppayil et al., 1996), elongation factor-1α protein genes (Shearer, 1995), β-tubulin genes (Li and Edlind, 1994), and orotidine 5′-monophosphate decarboxylase genes (Radford, 1993) have been used in fungal studies. However, none of these, with the exception of chitin synthase was PCR-mediated, as universal primers have not yet been developed for most of these genes.

7.3.2 PCR–RFLP

RFLP analysis has been a useful tool in phylogenetic studies with a variety of organisms (Michelmore and Hulbert, 1987; Bruns et al., 1991). RFLP of amplified fragments (PCR–RFLP) is now widely used for both fungal phylogeny and taxonomy (Chen, 1992; Liu and Sinclair, 1992; Bernier et al., 1994; Bunyard et al., 1994, 1996; Hopple and Vilgalys, 1994; Appel and Gordon, 1995; Donaldson et al., 1995; Erland, 1995; Harrington and Wingfield, 1995; Sappal et al., 1995; Buscot et al., 1996; Chiu et al., 1996; Edel et al., 1996; Guillemaud et al., 1996; Waalwijk et al., 1996; Leal et al., 1997). PCR–RFLP is a simple and inexpensive method compared with traditional RFLP or sequence analyses as it avoids the need for blotting, probing and/or sequencing. ITS or IGS regions have mainly been used for PCR–RFLP as these regions are highly polymorphic and can be easily amplified by PCR using universal primers.

7.3.3 RAPD and AP-PCR

Random amplification of polymorphic DNA (RAPD; Williams et al., 1990) or arbitrarily primed polymerase chain reaction (AP-PCR; Welsh and McClelland, 1990) is a simple and rapid method for detecting genetic diversity. Genetic diversity is assessed by amplification at low stringency with a single short primer of arbitrary sequence. The technique has been used to detect genetic variations among strains or isolates within a species (Goodwin and Annis, 1991; Cooke et al., 1996; Hseu et al., 1996; Boyd and Carris, 1997; Jeng et al., 1997; Maurer et al., 1997; Pei et al., 1997; Jungehülslng and Tuzdyski, 1997). The amplification conditions need to be rigorously standardized in order to obtain reproducible and consistent results, as the fragment pattern obtained is highly sensitive to concentrations of Mg²⁺, DNA polymerase, primers and template DNAs, and to cycling temperatures (Bruns et al., 1991).

7.4 PCR-derived Phylogenetic Studies

7.4.1 Broad ranging studies

Bruns et al. (1992) used 18S rDNA sequences to investigate evolutionary relationships within the fungi. The inferred tree topologies were in general
agreement with traditional classifications in that the *Chytridiomycota* and *Zygomycota* were recovered as basal groups within the kingdom, the *Ascomycota* and *Basidiomycota* appeared as a derived monophyletic group, relationships within the *Ascomycota* were concordant with traditional orders and divided the hemi- and euascomycetes into distinct lineages, and the *Basidiomycota* was divided into two groups, the holobasidiomycetes and phragmobasidiomycetes.

Berbee and Taylor (1993, 1995) calibrated the rate of 18S rDNA sequence change by relating the normalized per cent substitution associated with phylogenetic events to fungal fossils, the ages of fungal hosts, and the ages of symbionts. Their calculations suggested that fungal 18S rDNA undergoes substitutions at about 1% per lineage per 100 million years. Based on these calculations these authors suggested that the terrestrial fungi diverged from the chytrids approximately 550 million years ago, and that ascomycetes split from basidiomycetes approximately 400 million years ago, after plants invaded the land. Similarly, mushrooms, many ascomycetous yeasts, and common fungi in the genera *Penicillium* and *Aspergillus* may have evolved after the origin of angiosperm plants, during the last 200 million years. Simon *et al.* (1993) used a different set of ecological and fossil calibrations to estimate the origin of the vesicular–arbuscular mycorrhizae at 353–462 million years ago.

Van der Auwera *et al.* (1994, 1995) and van der Auwera and de Wachter (1996) studied the phylogenetic relationship of oomycetes, hyphochytriomycetes, and chytridiomycetes based on the sequences of rDNA including the 28S rRNA gene from *Phytophthora megasperma*, *Hyphochytrium catenoides*, and *Blastocladiella emersonii*. Their results showed: (i) the hyphochytriomycetes formed a monophyletic group with the oomycetes and heterokont algae, and that they were probably the closest relatives of the oomycetes, and (ii) that the chytridiomycetes were true fungi and not protists. Within the fungal clade, *B. emersonii* formed the first line of divergence, and the oomycete and hyphochytriomycete cluster appeared as a sister group of the fungal clade. These authors suggested that a possible common ancestor of the fungi, straenopiles, and alveolates, may have been a zoosporic fungus, which would mean that zoosporic fungi were paraphyletic instead of polyphyletic as previously suggested.

### 7.4.2 Filamentous ascomycetes

Nishida and Sugiyama (1993) sequenced the 18S rDNA from *Taphrina wiesneri* and *Saitoella complicata* in order to determine the phylogenetic placement of the major groups of higher fungi. They suggested that there were at least two major evolutionary lineages in the ascomycetes, and that *T. wiesneri* and *S. complicata* form a monophyletic branch that diverged prior to the separation of other ascomycetes. Nishida and Sugiyama (1994) sequenced the 18S rDNA from *T. populina* and *Protomyces lactucae-debilis*.
and compared these sequences with 75 sequences from other ascomycetes. Their phylogenetic analysis divided the Ascomycota into three major lineages: the hemiascomycetes, the euascomycetes, and a new line which included Taphrina and Protomyces, which they called the archiascomycetes.

Eurotiales and related species
The genera Penicillium and Aspergillus have been the subject of several phylogenetic analyses (LoBuglio et al., 1993, 1994; Peterson, 1993; Pitt, 1993; Verweij et al., 1995). Berbee et al. (1995) used 18S, 5.8S rDNA and ITS sequences to test whether Penicillium was monophyletic and to examine its relationship with the ascomycete family Trichocomaceae. Their results showed that Penicillium was not a monophyletic genus. The phylogenetic trees constructed by them showed Eupenicillium javanicum (which has a Penicillium anamorph) grouping with Neosartorya fischeri and Eurotium rubrum (which have Aspergillus anamorphs), rather than with Talaromyces flavus var. macrosporus or Talaromyces bacillisporus, two species with Penicillium anamorphs. Conversely, the genus Aspergillus was recovered as an apparently monophyletic group.

Phylogenetic relationships between strictly mitotic species in the genus Penicillium and Aspergillus, and closely related meiotic species have been considered based on sequences from the mitochondrial small subunit rDNA, and the nuclear ITS and 5.8S rDNA (LoBuglio et al., 1993; Geiser et al., 1996). In both cases meiotic and strictly mitotic taxa were often recovered clustered together, indicating that multiple independent losses of teleomorphs had occurred in both genera.

Verweij et al. (1995) investigated the phylogenetic placement of four species of Aspergillus based on 18S rDNA sequences. The species sequenced showed a very close intergeneric relationship to species of the genera Paecilomyces and Penicillium, and also to Eurotium rubrum and Monascus purpureus.

18S rDNA sequences were used by Bowman et al. (1992, 1996) to investigate possible relationships between four species of human pathogenic fungi placed in the Onygenales, and seven non-pathogenic fungi that were thought to be their nearest relatives on the basis of morphological features. The result showed that the pathogens were interspersed among the non-pathogenic fungi, which suggested that pathogenicity had arisen multiply within this group. The phylogenetic relationship of several human pathogenic fungi in Onygenales was also investigated by Harmsen et al. (1995).

Pyrenomycetes
There are two main views on the classification of the Clavicipitales within the unitunicated perithecial ascomycetes. One is that the Clavicipitales is a sister group to the Hypocreales, and the other is that it is a member of, or a near relative to, the Xylariales. The 18S rDNA sequences support the placement
of the Clavicipitales as a monophyletic sister group to the Hypocreales (Spatafora and Blackwell, 1993).

Acremonium is generally considered to be a highly polyphyletic form-genus which includes the clavicipitaceous grass endophytes. Glenn et al. (1996) used 18S rDNA sequences to investigate relationships within Acremonium, and they found that the genus was polyphyletic, with affiliations to at least three ascomycetous orders. Most of the species examined from the sections Acremonium, Gliomastix, and Nectrioidea appeared related to the Hypocreaceae, and the grass endophytes of sect. Albolanosa, and other taxa from the Clavicipitaceae formed a monophyletic group derived from within the Hypocreales. The thermophilic Acremonium alabamense, however, appeared to be derived from within the Sordariales. In order to eliminate some of the heterogeneity within Acremonium, while also emphasizing the unique biological, morphological, and ecological characteristics of the grass endophytes, they proposed that the anamorphs of Epichloë and closely related asexual grass endophytes be reclassified into the new form-genus Neotyphodium. Within the genus Epichloë, Schardl et al. (1991) used sequences of the ITS regions to examine relationships between E. typhina and its anamorphs, including endophytic mycosymbionts of various grasses of the subfamily Pooideae. These results indicated that the non-pathogenic endophytes have not necessarily co-evolved with their host species and that they arose from E. typhina on multiple occasions.

Rehner and Samuels (1994) used eukaryotic-specific divergent regions of 18S rDNA to investigate the phylogeny of Gliocladium. Their results indicated that Gliocladium was polyphyletic and that G. penicillioides, G. roseum, and Trichoderma virens (G. virens) were genetically distinct. Rakotonirainy et al. (1994) used two divergent regions of 28S rDNA to investigate phylogenetic relationships within the genus Metarhizium, one of the most ubiquitous entomogenous fungal genera. The results obtained from sequence analysis were also confirmed by isoenzyme polymorphisms.

Partial sequences of 18S and 28S rDNA were used by Seifert et al. (1997) to suggest that Spicillum roseum and Trichothecium roseum formed a single monophyletic group allied to the Hypocreales, although they found sister group relationships to be unclear.

Phylogenetic relationships within four species of Phialophora were considered by Yan et al. (1995) from RFLPs and sequence analysis of the 5.8S and ITS rDNA of 51 isolates. Clustering based on similarity values produced groups that were consistent with morphological species designations in each case. Bryan et al. (1995) assessed the relatedness of a number of Gaeumannomyces and Phialophora isolates by comparison of 18S, 5.8S rDNA and ITS sequences. They found that G. graminis var. tritici, G. graminis var. avenae, and G. graminis var. graminis could be distinguished from each other by nucleotide sequence differences in the ITS regions. The G. graminis var. tritici isolates can be further subdivided into R and N isolates [correlating with ability (R) or inability (N) to infect rye]. Isolates of Magnaporthe grisea
included in the analysis showed a surprising degree of relatedness to members of the *Gaeumannomyces–Phialophora* complex.

Hausner *et al.* (1993) used partial 18S and 28S rDNA sequences in a phylogenetic analysis of *Ophiostoma*. 18S rDNA sequence data suggested that *Ophiostoma* should remain the sole genus of the *Ophiostomataceae*, although there was not sufficient information for parsimony analysis to generate tree topologies with consistently high levels of statistical support. The sequences of the 5′ terminus of the 28S rDNA were also determined to examine phylogenetic relationships within the genus, and these data failed to support the subdivision of the genus based on either ascospore characters or the nature of the anamorph.

Sherriff *et al.* (1994) used 28S rDNA and ITS sequences to investigate the relatedness of a range of isolates of *Colletotrichum*, selected to represent the major morphological forms of the genus. They found that the species investigated could be divided into two groups. The first group, consisting of *C. lindemuthianum*, *C. malvarum*, *C. orbiculare*, and *C. trifolii*, was distinct from all the other species, and their DNA was highly homologous. They concluded that this group may represent a single species. The second group consisted of isolates of *C. gloeosporioides* which showed greater divergence in their DNA sequences than those of the first group, and it was concluded that isolates included within *C. gloeosporioides* represented more than one species. Sherriff *et al.* (1995) also compared sequences of ITS2 regions of isolates of *C. graminicola* from maize, sorghum and *Rottboellia*. The results indicated that the isolates from maize represented a species which was distinct from the isolates obtained from sorghum and *Rottboellia*. They recommended that the isolates from maize should be regarded as *C. graminicola*, and that those from sorghum and *Rottboellia* should be considered *C. sublineolum*.

Guadet *et al.* (1989), Peterson (1991) and O’Donnell (1993) investigated the phylogenetic relationships of *Fusarium* species using partial sequences of the 28S rDNA. Both Guadet’s and O’Donnell’s data showed *Gibberella* to be monophyletic, and O’Donnell suggested that *Nectria* was paraphyletic and that *Fusarium* was polyphyletic. O’Donnell (1992) also showed that there was a high degree of intraspecific variation in the ITS sequences of *F. sambucinum*. Edel *et al.* (1996) characterized 87 strains belonging to 18 species of *Fusarium* by PCR–RFLP analysis of ITS regions and variable domains of the 28S rDNA. Data from the RFLP analysis showed 23 rDNA haplotypes among the strains investigated. The groupings obtained by the restriction analysis were, on the whole, in agreement with other molecular and morphological classification criteria.

Waalwijk *et al.* (1996a, b) compared 13 species from *Fusarium* sections *Elegans*, *Liseola* and *Dlaminia* using the DNA sequences and RFLPs of the two ITS regions. These studies showed that although the ITS1 data were similar for most species (except *F. beomiforme* and *F. polyphialdicum*), species grouping based on the ITS2 sequences formed two distinct clusters.
that did not coincide with the section boundaries. O’Donnell and Cigelnik (1997) used a cladistic analysis of sequences obtained from multiple unlinked loci to consider the evolutionary history of the Gibberella fujikuroi complex. Gene phylogenies inferred from the mitochondrial small subunit rDNA, nuclear 28S rDNA, and the β-tubulin gene were generally concordant, providing strong support for a fully resolved phylogeny of all biological and most morphological species. However, a discordant gene tree was obtained from the ITS2 region. Ingroup taxa within the ITS2 gene tree formed two groups, designated type I and type II as reported by Waalwijk et al. (1996a). Interestingly, sequence analysis demonstrated that every strain tested from the ingroup species had both types of ITS2. Only the major ITS2 type, however, was discernible when PCR products were amplified and sequenced directly with conserved primers, and the minor ITS2 type was only recovered when ITS2 type-specific primers were used. Distribution of the major ITS2 type within the species lineages showed a homoplastic pattern of evolution, and so obscured true phylogenetic relationships. O’Donnell and Cigelnik (1997) suggested that the ancestral ITS2 types may have arisen following an ancient interspecific hybridization or gene duplication which occurred prior to the evolutionary radiation of the G. fujikuroi complex.

Appel and Gordon (1995, 1996) used RFLP analysis and partial sequences of the rDNA IGS region to investigate relationships among isolates of Fusarium oxysporum, including F. oxysporum f. sp. melonis and non-pathogenic strains. They found that the IGS haplotype and sequence correlated with vegetative compatibility group (VCG) and mtDNA haplotype in most isolates within F. oxysporum f. sp. melonis, but could not be used to differentiate among races. However, a race 1 isolate which was associated with VCG 0131 had greater affinity with VCG 0134 based on both mtDNA and IGS haplotype. Two IGS sequence types were found in this race 1 isolate, one suggesting an affiliation with VCG 0131 and the other similar to isolates in VCG 0134. Appel and Gordon (1996) suggested this result may indicate past somatic or sexual interactions between F. oxysporum f. sp. melonis, VCGs 0131 and 0134. Non-pathogenic isolates that were vegetatively compatible with the pathogenic ones were not closely related to the pathogen based on IGS sequence data, and so non-pathogenic and pathogenic isolates may share common alleles at vegetative compatibility loci by coincidence rather than because of recent clonal derivation from a common ancestor.

Discomycetes
Evolutionary relationships of apothecial ascomycetes (discomycetes) have been examined from 18S rDNA sequences (Gargas and Taylor, 1995). They obtained a most parsimonious tree that supported the monophyly of the orders Pezizales, Leotiales, and Lecanorales. However, there was no support for monophyly of the representative Caliciales.

Gargas et al. (1995) reported the presence of insertions (group I introns) in the nuclear small subunit rDNA of lichen-forming fungi. Of the 11
insertion positions they reported, six positions were known only from the lichen-forming fungi. They considered the possible phylogenetic significance and distribution of insertions in eukaryotic organisms.

O’Donnell et al. (1997) examined the phylogenetic relationships among ascomycetous truffles and true and false morels based on sequences from 18S and 28S rDNA. Parsimony analysis of the combined data set gave a single most parsimonious tree, indicating that the hypogeous ascomycetous truffle and truffle-like taxa studied represent at least five independent lineages within the Pezizales.

Phylogenetic relationships between the genera Plicaria and Peziza have been examined from sequences of the rDNA ITS1 region, the divergent D1 and D2 domains of the 28S subunit, and a region near the 5′ terminus of the 18S subunit (Norman and Egger, 1996). Parsimony analysis showed that Plicaria was a monophyletic group. However, several Peziza taxa that shared a number of morphological characters with Plicaria were closely related. The authors suggested that Plicaria should be recognized as a separate genus, although Peziza is paraphyletic if Plicaria is maintained. A phylogenetic analysis has also been made of Sarcoscypha species (Sarcoscyphaceae, Pezizales), based on ITS sequences (Harrington and Potter, 1997).

Carbone and Kohn (1993) sequenced the ITS1 region from 40 isolates of Sclerotiniaceae and three outgroup isolates in Leotiaceae in a study of the phylogenetic relationships among the Sclerotiniaceae. Their results supported their hypothesis that there was a sclerotial lineage, and suggested that this lineage had evolved relatively recently. However, they could not resolve the phylogenetic relationships among the non-sclerotial taxa due to their highly divergent ITS1 sequences.

Loculoascomycetes
Berbee (1996) sequenced the 18S rDNA of 16 species from seven families in the loculoascomycete orders Pleosporales, Dothideales, and Chaetothyriales in an examination of evolution within the order.

Leptosphaeria maculans is an important plant pathogen and isolates from Brassica spp. can be divided into two pathotypes: highly virulent (HV) and weakly virulent (WV) (McGee and Petrie, 1978). In addition to pathogenicity, HV and WV isolates differ in cultural characters and toxin production (Koch et al., 1989), and are also distinguishable by RAPD (Goodwin and Annis, 1991; Schäfer and Wöstemeyer, 1992), RFLPs (Koch et al., 1991), and electrophoretic karyotyping (Taylor et al., 1991). Xue et al. (1992) and Morales et al. (1993) independently sequenced the 5.8S rDNA and ITS regions from HV and WV isolates. Although the 5.8S rDNA sequences were identical between the HV and WV isolates, both ITS sequences were very divergent. Morales et al. (1993) showed that each pathogenicity group was statistically different from each other and that the WV isolates were more closely related to the Thlaspi isolates than to the HV isolates. Xue and Goodwin (1994) found insertions in domain V of the mitochondrial large
rDNA of *L. maculans*. Most of the inserted sequence was highly homologous (90%) between pathotypes, indicating that they were closely related, but the insertion in the WV pathotype had an additional 49 bp at the 3′ terminus of the insert. The 49 bp region contained a repetitive 7 bp sequence and had a higher GC content than the rest of the insert. The phylogenetic relationships among several *Leptosphaeria* species have been determined from the sequences of the 18S and 5.8S rDNA and ITS regions (Morales *et al.* 1995), and Khashnobish and Shearer (1996) used both morphological characters and ITS2 and partial 28S rDNA sequences to investigate phylogenetic relationships in some *Leptosphaeria* and *Phaeosphaeria* species. They showed that the *Phaeosphaeria* species formed a natural group while *Leptosphaeria sensu lato* was paraphyletic.

Jasalavich *et al.* (1995) investigated the phylogenetic relationships between three *Alternaria* species pathogenic to crucifers, *A. alternata*, and *Pleospora herbarum* from the sequences of 18S and 5.8S rDNA subunits and the ITS regions. They showed that all of the *Alternaria* species were closely related, and that *Pleospora* also appeared to be more closely related to *Alternaria* than to *Leptosphaeria*. Kusaba and Tsuge (1995) sequenced the ITS of some *Alternaria* species, including seven that were known to produce host-specific toxins. Phylogenetic analysis of the sequence data by the neighbour-joining method showed that the seven toxin-producing fungi formed a monophyletic group together with *A. alternata*. Conversely, the species of *Alternaria* that were clearly distinguishable from *A. alternata* by morphological features were also clearly separated from *A. alternata* on the basis of their ITS sequences. Based on these findings, and previous work (Kusaba and Tsuge, 1994), the authors suggested that isolates of *Alternaria* which produced host-specific toxins were pathogenic variants of the single variable species *A. alternata*.

Other filamentous ascomycetes
The *Erysiphales* (powdery mildews) have been classified in both the *Pyrenomycetes* and the *Plectomycetes* on the basis of their morphological features. However, analysis of their 18S rDNA sequence has shown that they are distinct from both groups (Saenz *et al.*, 1994).

Phylogenetic relationships of the imperfect mycorrhizal fungus *Cenococcum geophilum* within ascomycetes have been considered from 18S rDNA sequences (LoBuglio *et al.*, 1996). Parsimony and distance analyses placed *C. geophilum* as a basal, intermediate lineage between the two Loculoascomycete orders, the *Pleosporales* and the *Dothidiales*, strongly suggesting that *Elaphomyces* was of Plectomycete origin. This result contradicted the original hypothesis that *Elaphomyces* was the closest relative of *C. geophilum*, and also suggested that there were at least four independent lineages of mycorrhizal fungi among the ascomycetes included in the study.

Nishida *et al.* (1993) demonstrated that there were two group I introns in the 18S rRNA coding region of *Protomyces inouyei* (archiascomycetes).
Comparison of the two introns of *P. inouyei* with those of other group I intron-containing higher fungi showed that intron A of *P. inouyei* was located in the same position as an intron in *Pneumocystis carinii*, while intron B occupied an insertion site also found in *Ustilago maydis*. They suggested these group I introns may be subject to horizontal transfer.

### 7.4.3 Basidiomycetes

Swann and Taylor (1993) sequenced the 18S rDNA from nine basidiomycetes and aligned them with ten other basidiomycetes and three ascomycete sequences. The phylogenetic tree showed that the basidiomycetes fell into three major lineages: the *Ustilaginales* smuts, simple septate basidiomycetes, and hymenomycetes which formed a basal unresolved trifurcation. Suh and Sugiyama (1994) also suggested that there were three lineages in the basidiomycetes.

**Agaricales**

Hibbett *et al.* (1995) investigated the phylogenetic relationships of the shiitake mushroom from ITS sequences. Their ingroup consisted of seven isolates of *Lentinus edodes*, nine isolates of *L. lateritia*, and five isolates of *L. novaezelandiae*. They found four independent lineages of shiitake in Asia–Australasia, and this provided some support for the morphologically based species concepts. There was a strong correlation between the geographic origins of the isolates within each lineage, and a biogeographic interpretation of the ITS tree suggested that the centre of origin for shiitake in the Asia–Australasia region was in the South Pacific. Chiu *et al.* (1996) studied the genetic diversity of cultivated strains of shiitake used in China using AP-PCR, RAPD and PCR–RFLP of ITS regions. The results indicated that cultivated strains of shiitake in China are genetically very homogeneous, very like cultivated strains of *Agaricus bisporus* and *Volvariella volvacea*.

Hibbett and Vilgalys (1993) also investigated the phylogenetic relationships of *Lentinus*, using morphological characters and sequences from the divergent regions of the 28S rDNA. They found that although *Lentinus sensu* Pegler was not monophyletic, the genus consisted of three separate monophyletic groups. These largely corresponded to *Neolentinus*, *Panus*, and *Lentinus sensu stricto*, with the latter apparently derived from the *Polyporaceae*, suggesting that lamellae are products of convergent evolution.

The confused taxonomy of the genus *Armillaria* has precluded a clear understanding of the biology of the major species. Anderson and Stasovski (1992) have used IGS sequences to investigate the phylogenetic relationships of several northern hemisphere species of *Armillaria*. The majority of northern hemisphere species were found to be closely related to one another, relative to *A. mellea* and *A. tabescens*. The majority of the northern hemisphere species were divided into two groups, one composed of *A.*
ostoyae, A. gemina and A. borealis, and the other consisting of A. lutea, A. calvescens, A. cepistipes, A. sinapina, species IX and species X.

Hinkle et al. (1994) determined the complete 16S-like rRNA coding regions of the fungal symbiont of five genera of attine (leaf-cutting) ants and two free-living fungi. The phylogenetic tree generated from this data placed the attine fungal symbionts as homobasidiomycetes in the order Agaricales. Comparison of the topology of the attine fungal symbiont phylogenetic tree with a tree based on attine ant morphology suggested that there had been a long co-evolution of leaf-cutting attine ants and their fungal symbionts.

Phylogenetic relationships within the coprinoid taxa and some close relatives have been studied from PCR–RFLP of nuclear large subunit rDNA and the ITS2 region (Hopple and Vilgalys, 1994). These authors suggested that the genus Coprinus was at least paraphyletic and probably polyphyletic and that Psathyrella was derived from within Coprinus. The results also suggested that the two secotioid taxa were closely related to Coprinus comatus and that Bolbitius was a sister genus to Coprinus.

Bunyard et al. (1996) used PCR–RFLP of the 26S and 5S rDNA, and the IGS between the 26S and 5S rDNA for a phylogenetic study of Agaricus species. They found that the most closely related species to A. bisporus were A. excellens, A. chionodermus and A. caroli.

Gastromycetes
ITS regions from 47 isolates belonging to 38 recognized species of Suillus sensu lato have been sequenced in a phylogenetic analysis using parsimony as well as distance methods (Kretzer et al., 1996). The results suggested that the genera Boletinus and Fuscoboletinus, that are often considered within S. sensu lato, were not monophyletic, and that isolates of Suillus granulatus derived from either North America or Europe and Asia are polyphyletic and may represent at least two different species. This study also showed that within Suillus sensu lato, mycorrhizal associations with Larix were a primitive association and that host changes to Pinus and Pseudotsuga occurred only once. Baura et al. (1992) used ITS sequences to propose that Gastrosuillus laricinus was a recent derivative of Suillus grevillei.

Aphyllophorales
The phylogenetic relationships within Ganoderma and the G. lucidum species complex have been considered from sequences of ITS regions and the divergent domain D2 of the 25S rDNA (Moncalvo et al., 1995a, b). Parsimony analysis of this data identified six lineages in the G. lucidum complex. It was suggested that each lineage represented one or more putative species, however, there was little correlation between rDNA gene phylogeny and morphology within the species complex. Hseu et al. (1996) also attempted to differentiate isolates of the G. lucidum complex using RAPD analysis. However, data from RAPDs did not distinguish the same clades as ITS and it seems likely that while ITS sequences can be used to identify
species within the *G. lucidum* complex, RAPDs can be used to differentiate between isolates which have identical ITS sequences.

PCR–RFLP analyses have been used for phylogenetic studies and species identification with several kinds of mushrooms and ectomycorrhizae. Gardes *et al.* (1991) considered variations in the length, RFLP, and primary sequence of the PCR-amplified ITS regions and mitochondrial large subunit rDNA ingroupings of ectomycorrhizal fungi, particularly in the genus *Laccaria*.

**Uredinales**
Relatively few molecular phylogenies have been proposed for the rust fungi. Zambino and Szabo (1993) used the sequences of ITS regions to investigate phylogenetic relationships among strains and *formae speciales* of *Puccinia coronata*, *P. graminis*, *P. recondita* and other cereal and grass rusts and related species. They showed that isolates of *P. graminis* and *P. coronata* from various hosts each consisted of a distinct cluster, but that strains of the *P. recondita* species complex isolated from various hosts did not. In comparisons among *formae speciales* of *P. graminis* and *P. coronata*, the *formae speciales* that had identical ITS sequences were those that were already known to be closely related by crossing, isozyme, and host range studies. However, in some cases the relationships among *formae speciales* based on sequence data contradicted the current taxonomy of the cereal rusts.

Chen *et al.* (1993) considered the relationship between variation in virulence and RAPD patterns in 115 single-uredospore isolates from 23 collections of *P. striiformis*. They found there was little apparent association between virulence and RAPD patterns, indicating that the RAPD polymorphisms were independent of virulence.

**Other basidiomycetes**
Eleven anastomosis groups (AG 1–10 and AG BI) have been recognized in *Rhizoctonia solani*, and 25 intraspecific groups (ISGs) have been recognized within these on the basis of isoenzymes and ITS polymorphisms (Liu and Sinclair, 1992, 1993; Liu *et al.*, 1993). Liu *et al.* (1995) found four major groups from RFLP analysis of 18S rDNA of 161 isolates of the 25 ISGs, and maximum parsimony and likelihood analyses showed that AG 10 isolates were distinct and only distantly related to the majority of the other AGs.

### 7.4.4 Zygomycetes

Phylogenetic studies on zygomycetes are comparatively rare compared with those on other fungal groups. Nagahama *et al.* (1995) determined the 18S rDNA sequences for four representative species of the *Entomophthorales* and the trichomycete *Smittium culisetae*. Their analysis included ten previously published reference sequences and showed that the seven zygomycete species investigated formed four clusters. The four entomophthoralean
species were recovered in two clusters, with *Basidiobolus ranarum* in one cluster with *Chytridium*, *Spizellomyces* and *Neocallimastix*, while the remaining three entomophoralean species, *Conidiobolus coronatus*, *Entomophthora muscae* and *Zoophthora radicans*, formed a second cluster with *Mucor. Glomus etunicatum* was found to be a basal clade to the ascomycetes and basidiomycetes, and *Smittium culisetae* was placed close to the divergence of *Entomophthorales* from the chytrid–*Glomus–ascomycete–basidiomycete* clade. The results also suggested that flagellae had been lost independently in several lineages in the tree and that there was considerable phylogenetic divergence within the chytrids.

*Geosiphon pyriforme* is the only known example of endocytobiosis between a fungus and a cyanobacterium. Gehrig *et al.* (1996) used 18S rDNA sequences to investigate the taxonomic and evolutionary relationships of *G. pyriforme* to fungi forming arbuscular mycorrhiza (*Glomales*). The *Glomales*, including *Geosiphon*, formed a single clade distinct from groups of zygomycetes and *G. pyriforme* was placed at the ancestral base of the *Glomales*. *Geosiphon* is able to form a ‘primitive’ symbiosis with a unicellular photoautotrophic organism, and based on their phylogeny the authors suggested that a hypothetical association of a Glomus-like fungus with a green alga may have occurred as a stage in the evolution of the land plants.

### 7.4.5 Oomycetes, Hyphochytriomycetes

The PCR–RFLP of the nuclear large subunit rDNA, ITS regions, and mitochondrial large subunit rDNA of three heterothallic *Pythium* species, *P. heterothallicum*, *P. splendens* and *P. sylvaticum*, were determined by Chen (1992). The ITS of both *P. heterothallicum* and *P. splendens* was about 850 bp long, and the ITS for *P. sylvaticum* was about 1020 bp long; each length variant showed distinct RFLP banding patterns. A phylogenetic analysis based on the variable restriction sites in the ITS region showed that the heterothallic species did not form a monophyletic group, suggesting that heterothallism does not represent a distinct species lineage in the genus.

Briard *et al.* (1995) determined the nucleotide sequences of a divergent region of 28S rDNA from 23 species of *Pythiaceae*, and compared these to assess phylogenetic relationships. Although a high level of diversity was found within *Pythium*, *Phytophthora* appeared to be very homogeneous. They found that there was a strict correlation between groups of species within *Pythium* defined by molecular taxonomy and those defined on the basis of the morphology of sporangia.

The sequences of the ITS regions from *Phytophthora* species have been independently determined by several authors. Lee and Taylor (1992) used ITS sequences in a phylogenetic study of five *Phytophthora* species: *P. palmivora*, *P. megakarya*, *P. capsici*, *P. citrophthora* and *P. cinnamomi*. Their results showed that cacao isolates of *P. capsici* and *P. citrophthora* were closely related, that *P. palmivora* and *P. megakarya* shared a common
lineage, and that *P. cinnamomi* was only distantly related to the other species. Crawford *et al.* (1996) used rDNA sequences in an evolutionary analysis of 15 *Phytophthora* species. Analysis of papillate, semi-papillate and non-papillate species showed that sporangium papillation had phylogenetic significance, with the three morphological groups each forming separate clusters. Within the *P. megasperma* species complex, separate evolutionary lines were identified for *P. medicaginis*, *P. trifolii* and *P. sojae*, species formerly regarded as a *formae speciales* of *P. megasperma*. This finding confirmed their recent reclassification as biological species. *P. macrochlamydospora* from soybean, which has only been observed in Australia, was found to be closely related to *P. sojae*, indicating a possible common ancestry. Cooke *et al.* (1996) used the RAPD analysis and rDNA sequences in a phylogenetic study of group I species of *Phytophthora*. The RAPD banding patterns separated *P. iranica* and *P. clandestina* from the other *Phytophthora* species, *P. idaei*, *P. pseudotsugae* and *P. cactorum*. Collar rot isolates from apple placed in *P. cactorum* clustered separately from straw-berry crown rot isolates, while isolates from raspberry appeared to have affinities with both clusters.

### 7.5 Conclusions

Our knowledge of fungal phylogeny is rapidly being increased by PCR-mediated studies. As described above, most of these studies have been based on nuclear or mitochondrial rRNA genes, because conserved rRNA coding regions and variable spacer regions are present within a rDNA repeat, which enables the design of universal primers suitable for a wide range of fungi. However, recently, the use of sequences obtained from protein genes has increased. Sequence data obtained from different DNA regions will be required in future to construct more precise phylogenetic trees. While most studies described in this chapter have focused on fungal systematics, molecular phylogenetic study is also useful to infer morphological, ecological and physiological evolution (Berbee and Taylor, 1995). Furthermore, if the genes directly related to morphological, ecological and physiological phenotypes were identified and sequenced, then comparative analyses of these genes would make it possible to investigate how these phenotypes have evolved.

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Applications in Fungal Phylogeny


PCR Applications to the Taxonomy of Entomopathogenic Fungi

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8.1 Introduction

The extent of biodiversity within fungi is often underestimated: there are over 72,000 described species and the total number of species in the world may be as many as 1.5 million (Hawksworth, 1995). It is more widely known that insects are a highly diverse component of the animal kingdom with large numbers of undescribed species and it is estimated that a total of 10 million species of insect exist. Given that 5% of these insects are attacked by a specific fungus then it might be expected that there are 500,000 species of fungi as symbionts, commensals or pathogens of insects (Hawksworth, 1991). The diversity of association between insects and fungi has recently been reviewed by Lawrence and Milner (1996) and Murrin (1996). While over 750 species of fungi have been described as being pathogens of insects, only a small proportion of these have been intensively studied, mainly because they attack pest insects and are, therefore, of interest as biological control agents. The different types of fungi which are pathogenic on insects and the detailed ecology of some of these have been reviewed by Glare and Milner (1991).

There are two basic approaches to the use of pathogens for biological control of insects. A pathogen can be introduced into an area where it does not naturally occur in the hope of achieving long-term classical biological control. The other strategy is to mass-produce the pathogen for repeated use in an area as a biological insecticide. In both cases, it is important to know the genetic make-up of the isolate of the fungus being released into the field. Increasingly scientists are finding that PCR methodologies are useful in these applied programmes. This increased knowledge of the genetic make-up of
isolates is being applied to track the distribution of artificially released isolates in the field, to understand the host specificity profile of an isolate and for protection of intellectual property. The obvious implications for a greater understanding of the taxonomy of the pathogens involved is often a side-benefit, and not a prime objective of the research.

In this chapter, we review the use of PCR for study of the taxonomy of entomopathogenic fungi and use as a case study our own research on *Metarhizium*. Finally we offer some conclusions and outline some of the future directions for this research. For a more general treatment of the applications of molecular biology to entomopathogenic fungi the reader is referred to Khachatourians (1996), and the application of biotechnology to the development of mycoinsecticides has been covered by Hegedus and Khachatourians (1995).

8.2 Recent Studies on PCR of Entomophthorales

The largest and most successful group of insect pathogenic fungi is the 150 or so species within the order *Entomophthorales* of the subdivision *Zygomycetina*. Most of these fungi were once placed in a single large genus, *Entomophthora*, but now some nine genera are generally recognized: *Conidiobolus, Entomophaga, Entomophthora, Eryniopsis, Neozygites, Erynia, Zoophthora, Strongwellsea* and *Massospora*. Some species can be grown on simple media and have been investigated as biological insecticides but most are too fastidious for this application. Unfortunately the species most effective as biological control agents are very widely distributed (along with their hosts) and so opportunities for introductions are rare.

Nagahama *et al.* (1995) examined the phylogenetic relationships between the entomophthoran genera, *Zoophthora, Entomophthora* and *Conidiobolus* and other zygomycetes such as *Mucor, Glomus, Smittium* and *Basidiobolus* by comparing sequences of the 18S RNA gene. Phylogenetic trees were developed using neighbour-joining (Saitou and Nei, 1987) or parsimony (Swofford, 1993) and the robustness of the branches was assessed using bootstrap analysis (Felsenstein, 1985). The two trees both showed that the three insect pathogenic genera were distinct but closely related; *Mucor* was the most closely related free-living genus. More studies of this type are needed to cover a wider range of genera and isolates. The results may help to clarify, for example, the controversial distinction between *Erynia* and *Zoophthora*.

Three species which have been introduced into other countries either accidentally or deliberately are *Entomophaga maimaiaga* into the USA (Andreadis and Weseloh, 1990), *Zoophthora radicans* into Australia (Milner *et al.*, 1982), and *Entomophaga grylli* into both Australia (Milner, 1985) and the USA (Carruthers and Onsager, 1993). PCR studies of all these species have been published in addition to those on the ubiquitous aphid pathogen *Erynia neoaphidis* (syn. *Pandora neoaphidis*).
The gypsy moth, *Lymantria dispar*, is an important pest of forests in the USA. In 1989, extensive mortality of caterpillars occurred in the north-east USA and the causative organism was provisionally identified as a member of the *Entomophaga aulicae* complex, an entomophthoran pathogen of a variety of species of Lepidoptera in North America. Hajek et al. (1990) used allozymes and RFLP to compare 20 isolates of *E. aulicae* from a variety of hosts in the USA and Canada, with six isolates of *E. maimaiga*, a morphologically similar fungus from the *L. dispar* in Japan, and three isolates of the new fungus from gypsy moth caterpillars infected in the north-east USA. Hajek et al. (1996b) have extended these studies using PCR products from two primers as well as RFLP used previously. rDNA primers for the 18S and 25S genes were used to amplify 200 bp of the 3′ end of the 18S gene and 1100 bp of the 5′ end of the 25S gene. The results supported the splitting of *E. aulicae* into three groups based on RFLP analysis (Walsh et al., 1990) and showed that a new isolate obtained from *Orygia vetusta*, a lymantriid closely related to the gypsy moth, belonged to group 2 *E. aulicae* and was distinct from *E. maimaiga* from the gypsy moth in the USA and Japan. Unfortunately the original host of the group 2 *E. aulicae* reference strain is not known for certain but is thought to be a *Heliothis* sp. These data provide strong evidence that there are four cryptic species within the *E. aulicae* complex in the USA which are ecologically separated by attacking different hosts. Only one of these species, *E. maimaiga*, has been characterized and named.

Recent PCR studies on two other apparently broad host-range entomopathogenic fungi, *Zoophthora radicans* and *Erynia neoaphidis*, have also demonstrated that these are species complexes. Hodge et al. (1995) used RAPDs to compare 38 isolates from a variety of hosts around the world. The aim of the study was to determine whether or not seven isolates of this fungus, originally from Serbia and field-released near Ithaca, New York (for biological control of the potato leafhopper, *Empoasca fabae*) had become established. The results gave a high level of correlation between isolates from Serbia and those subsequently isolated from field-release sites at Ithaca. More interesting was the finding that aphid-derived isolates were distinct from both the coccidid isolates and from isolates derived from Lepidoptera. In all some ten groups could be distinguished on the basis of RAPD patterns. Further work is needed to determine the taxonomic implications of these results. They support previous studies which proposed host-specific groups within *Z. radicans* (Milner and Mahon, 1985; Glare et al., 1987). The successful introduction of a strain of *Z. radicans* into Australia from Israel for the control of the spotted alfalfa aphid, *Theroaphis trifolii f. maculata*, was undertaken on the understanding that isolates already present in Australia on Lepidoptera, Diptera and Hymenoptera, were unable to attack aphids (Milner et al., 1982).

The relationships within *Zoophthora* spp. were further investigated by Hajek et al. (1996a) who investigated the origin of an outbreak of *Zoophthora*
phytonomi in North American alfalfa weevils, *Hypera punctata*. Similar methods, using RAPDs, were used to those of Hodge *et al.* (1995). The results clearly delineated two distinct groups within *Z. phytonomi*, both of which were distinct from two isolates of *Z. radicans* from a lepidopteran and a homopteran which gave identical patterns. The authors postulated that one of the two genotypes may be ‘new’ to North America, either because of importation from Europe or through genomic change (e.g. by mutation). They found that spore size, traditionally a key taxonomic character, was too variable to be of any taxonomic value.

In a similar study, Rohel *et al.* (1997) compared RAPD patterns for 31 isolates of the *Erynia neoaphidis* complex. As with the *E. aulicae* complex, only a single species *Er. kondoiensis* had been formally recognized and described as a distinct species. *Er. kondoiensis* was distinguished from *Er. neoaphidis* on the basis of allozymes, spore size and ecological factors (Milner *et al.*, 1983). Rohel *et al.* (1997) showed that the *Er. neoaphidis* complex clearly displays size polymorphism in the internally transcribed spacer (ITS) region. RAPD data showed that there were four major groups within the 31 isolates studied. The majority of isolates, which were from aphid hosts in France and other countries, were placed in group 1100, two isolates from non-aphid hosts in Mexico were group 575, the *Er. kondoiensis* isolate from *Acyrthosiphon kondoi* in Australia plus two other isolates from *Aphis* sp. in Mexico and *Acyrthosiphon pisum* in Brazil constituted group 1000, and seven isolates all from aphids in France constituted group 1450. Both groups 1000 and 1450 had smaller conidia than the (probably ‘typical’) *Er. neoaphidis* group 1100, and they grew more rapidly on artificial media. Further ecological and molecular studies are needed to determine the differences between these groups, especially groups 1100 and 1450, both of which occur on a range of aphid species in France. The two isolates from non-aphid hosts are likely to constitute a distinct species.

### 8.3 Recent Studies on PCR of Ascomycota

A large number of species of fungi from the *Ascomycota* have been described as pathogens of insects including the genera *Cordyceps*, *Torrubiella* and *Nectria*. Recent molecular research has focused on the *Ascospaera* spp. which cause the disease known as chalkbrood in bees (Bissett *et al.*, 1996). Lu *et al.* (1996) found that RAPD patterns could be used to detect the infection in leafcutter bees and to distinguish between bees infected with *A. aggregata* and those infected with *A. larvis*. They reported that a quick ‘vortexing’ method for extracting DNA was effective for use in RAPD analysis. All the primers except one gave clear banding patterns and these were consistent between infected cadavers and those from pure cultures; while uninfected insects gave quite different patterns. There were obvious differences in banding patterns between insects infected with either species of *Ascospaera*
and non-spore-forming cadavers gave banding patterns similar to those from spore-forming cadavers. Lu et al. (1996) concluded that this method provides a rapid and accurate identification of the infection in leafcutter bees and that species-specific ITS primers would provide a more reliable way of recognizing species.

Recently, five new species of *Ascosphaera* have been described from bee material in Australia, using traditional taxonomic methods (Anderson and Gibson, 1997), taking the total of known species to 25. They also compared 20 of these species using sequence analysis of the ITS1 and ITS2 regions (Anderson et al., 1997). The methodology used was essentially that adopted for *Metarhizium* by Curran et al. (1994). Sequences were found to be remarkably uniform within species and small differences between species were therefore considered important and diagnostic. The ITS1 region was found to be more variable than the ITS2 region. Four distinct groups were detected which correlate with morphological data. For example, the eight species clustered with *A. apis* all have large, blackish spore cysts, with ellipsoidal to subcylindrical ascospores that are small or of average size. Because of the limited differences in morphology, the molecular data were used as the primary character for separating some species, e.g. *A. subcuticulata* from *A. aggregata*. Interestingly, these clusters did not correlate with the host taxonomy, though there was some evidence that the saprophytic/parasitic life cycle correlated with these clusters. The results of Anderson et al. (1997) also support the conclusions of Berbee and Taylor (1992, 1995) who found, using sequence data from the nuclear 18S rDNA gene, that *Ascosphaera* and *Eremascus* are closely related genera.

### 8.4 Recent Studies on PCR of Mitosporic Fungi

#### 8.4.1 Verticillium spp.

One of the first fungi to be commercialized as a mycoinsecticide was *Verticillium lecanii* for control of aphids (trade-named Vertalec) and for whiteflies (trade-named Mycotal). Various species of insect pathogenic fungi were synonymized under the name *V. lecanii* by Gams (1971). Jun et al. (1991) tested the validity of this revision using a range of morphological, physiological and biochemical characters to establish the taxonomic relationships between 64 isolates of *Verticillium* spp. They found that isolates of *V. lecanii* clustered according to their source and suggested that further work using enzyme patterns and DNA composition might support the case for separating entomopathogenic, fungicolous and plant-pathogenic isolates into distinct taxa. Typas et al. (1992) used RFLPs and found that three isolates of *V. lecanii* from aphids (two species) and whitefly (one species) clustered together and were distinct from six plant pathogenic species. Roberts et al. (1995) used RAPDs to compare *V. lecanii* with plant pathogenic species *V.*
albo-atrum and V. dahliae and concluded that V. lecanii was highly variable and quite distinct from the other two Verticillium species. Other workers such as Nazir et al. (1991) have used sequence data of the ITS region to differentiate between species and strains within plant-pathogenic members of the genus. These data suggest that V. lecanii could be incorrectly named and that it could be separated into a number of varieties/species which were ecologically distinct.

Mor et al. (1996) analysed 36 isolates, mostly from insects and identified as V. lecanii, using RAPDs. Unlike Jun et al. (1991) the isolates did not cluster according to source, nor was there any correlation with geographical location or virulence for the whitefly, Bemisia tabaci. They conclude ‘it appears that virulence determinants of V. lecanii are also unrelated to polymorphic DNA pattern as expressed by RAPDs’.

8.4.2 Paecilomyces spp.

Two species of Paecilomyces, P. farinosus and P. fumosoroseus, are important as natural control agents of insects and are being evaluated as potential mycoinsecticides. A third species, P. lilacinus, is used as a biological control agent for plant parasitic nematodes in the Philippines.

Tigano-Milani et al. (1995b, 1995c) have been investigating variation within these species using RAPDs. The first of these studies (Tigano-Milani et al., 1995a) was concerned with P. lilacinus and compared the banding patterns of 28 isolates identified as P. lilacinus, mainly from soil and nematode material in Brazil, with the banding patterns of P. fumosoroseus (two isolates), P. farinosus (three isolates) and P. amoenoroseus (one isolate). The polyacrylamide gels showed considerable variation within P. lilacinus and enabled a large number of bands to be scored resulting in 293 scorable characters. Cluster analysis with P. fumosoroseus as the outgroup gave a large number of clusters with two isolates, code-named CG301 and CG190, being particularly divergent. The tRNA phylogenic tree gave a smaller number of clusters based on the 112 scorable characters. Interestingly, one isolate of P. farinosus clustered in the middle of the P. lilacinus isolates while CG190 was clustered with an isolate of P. amoenoroseus and one of P. farinosus. It was concluded that P. farinosus was not monophyletic. However apparently no attempt was made to determine whether or not CG190, isolated from the soil, was pathogenic for insects. While these results clearly indicate a high degree of variability within P. lilacinus and suggest that polyacrylamide gels are potentially useful for genetic fingerprinting of strains, the phylogenetic conclusions are open to question. Do the tRNA genes have adequate information content to allow the drawing of phylogenetic conclusions? Were the closely related strains of P. fumosoroseus suitable as outgroups?

Identical methods were applied by Tigano-Milani et al. (1995b) to study 27 P. fumosoroseus isolates, of which 15 came from B. tabaci, in comparison with one strain of P. lilacinus and nine strains of Paecilomyces not assigned
to species. As with *P. lilacinus*, the RAPD patterns revealed a high degree of genetic divergence between strains. Cluster analysis placed the *P. fumosoroseus* isolates into three groups. Group 1 isolates were morphologically similar and resembled the typical *P. fumosoroseus*, and group 2 isolates were more polymorphic and this group contained most of the *B. tabaci* isolates. Group 1 included isolate CG170 derived from the PFR-97 pilot product being produced by ECO-tek in the USA. The group 3 isolates were highly divergent from what can be regarded as normal. This is similar to the situation in *Metarhizium* where it is also a species aggregate (see case study below). The phylogenetic analysis based on the tRNA primers did not clarify the situation because group 1 and group 2 isolates often clustered together, again suggesting that the data may be unsuitable for this type of analysis.

8.4.3 *Beauveria* spp.

Agostino Bassi is credited with being the first scientist to demonstrate that a disease was caused by a microorganism, when in 1835 he infected silkworms with *Beauveria bassiana*. Since that time, members of the genus *Beauveria* have been intensively studied all over the world. A diverse range of isolates sharing the essential features of *Beauveria* have been described and some six species are generally recognized at present: *B. bassiana*, *B. brongniartii*, *B. amorpha*, *B. vermicomia*, *B. velata* and *B. caledonica* (Glare and Inwood, 1997). Distinguishing features of *Beauveria* include conidiophores which produce one-celled conidia in whorls or dense clusters of sympodial, short or globose or flask-shaped conidial cells with apical denticulate rachi. Generally, isolates are insect pathogens which produce cadavers covered in white mycelium and conidia sometimes forming pronounced coremia. *B. calendonica*, described from moorland soil in Scotland (Bissett and Widden, 1988), is the only species not known to be pathogenic to insects and may be entirely saprophytic.

Prior to the development of PCR, Rakotonirainy et al. (1991) sequenced a 500 bp segment of the 28S RNA gene of nine isolates of *B. bassiana* and compared them with *Fusarium* and *Tolypocladium*. The data confirmed that these *Tolypocladium* should not be synonymized with *Beauveria*. Kosir et al. (1991) used RFLP banding patterns to differentiate two isolates of *B. bassiana*, one a virulent isolate and the other a derived mutant of much lower virulence. Pfeifer et al. (1993) analysed a mitochondrial gene of *B. bassiana* isolate GK2016 using restriction enzymes, gene probe hybridization and DNA sequence comparisons. They found the mtDNA to be circular and 28.5 kbp in length. By using probes from other organisms, they were able to produce a restriction enzyme map. They were unable to find any introns and concluded that the sequence was more similar to that of *Aspergillus nidulans* than to two other species, *Podospora anserina* and *Neurospora crassa*.

The first application of PCR was by Bidochka et al. (1993) who compared RAPD patterns of 24 isolates of *Metarhizium* and *Beauveria* from
grasshoppers. Using three primers and analysing the data by means of the method of Nei and Li (1979), they found considerable variability within *M. anisopliae* but 12 ‘acridid’ isolates clustered together and showed much less variability. These were referred to as *M. flavoviride* because they included ARSEF 2023, an isolate described as *M. flavoviride var. minus* by Rombach *et al.* (1986) (see below). Isolates of the two *Metarhizium* species were found to be more similar to each other than to *B. bassiana*. This study clearly showed the potential of RAPDs for identifying species and strains of entomopathogenic fungi.

Contrasting approaches to using RAPDs for studying variation within *Beauveria* are shown in recent papers: Maurer *et al.* (1997) have confirmed the existence of host-dependent clusters while Glare and Inwood (1997) have found evidence of a distinct New Zealand group of *B. bassiana* isolates. The 36 isolates studied by Maurer *et al.* (1997) were all classified as *B. bassiana* on the basis of conidial morphology and were derived from pyralids (mostly the European corn borer, *Ostrinia nubilalis*), curculionids (mostly *Sitona* spp.) and four isolates from chrysomelids. Isolates were from Europe, Latin America, South America and Africa. RAPD and RFLP analyses were used and the isolates were bioassayed against European corn borer and the weevil *S. lineatus*. The results for the European corn borer isolates were particularly convincing. Twelve of the 13 most virulent isolates originated from this insect, and when analysed they clustered together with the one exception: isolate Bb231 from *Sitona*, which clustered closer to the corn borer isolates and was only slightly pathogenic for *S. lineatus*. The curculionid isolates were more variable but also tended to cluster together. This study shows that genetic fingerprinting, using RAPDs or a similar method, can be a powerful tool for screening isolates to detect those highly virulent for a particular pest. Maurer *et al.* (1997) also compare two dendograms produced for the RAPD and RFLP data using an unweighted pair-group algorithm and found that these dendograms showed a remarkable degree of congruity. However, the authors correctly point out problems of using a hierarchical method for placing isolates in groups with implied relatedness.

The difficulties posed by identifying isolates of *Beauveria* based on morphological characters led Glare and Inwood (1997) to use molecular tools to compare isolates from New Zealand with those from other countries. They compared 26 isolates, most of which were classified as *B. bassiana* or *B. brongniartii*, on the basis of conidial morphology. Other isolates studied were single isolates of *B. amorpha*, *B. caledonica* and *B. velata* of the ARSEF collection, one isolate with curved conidia from Chile referred to as *B. vermiconia* and an isolate of *Akanthomyces* sp. from Australia which was used as an outgroup in the analysis. DNA was extracted and analysed by RAPDs and by RFLPs of the ITS region. Neither RAPD patterns nor RFLP banding from the ITS region separated out *B. bassiana* and *B. brongniartii*. The RAPD analysis provided many more differences in scorable bands and using these data three clusters were apparent: *B. bassiana* from New Zealand
and other countries plus two overseas B. brongniartii, a second cluster containing only New Zealand isolates of B. bassiana, and a final cluster containing only B. brongniartii from New Zealand and other countries. The other four species clustered together with the Akanthomyces sp. outgroup. However, as discussed by Maurer et al. (1997), it is difficult to draw phylogenetic conclusions from this type of analysis. The RFLP patterns gave less variation but supported the conclusion that there was a ‘New Zealand’ genotype of B. bassiana which clustered with B. amorpha, B. caledonica and B. vermiconia. The second major cluster contained all the other B. bassiana plus B. brongniartii isolates. An interesting speculation in this paper is that the two genetically distinct groups of B. bassiana found in New Zealand represent an indigenous (‘New Zealand’) form and a post-European settlement group. It is also interesting that B. brongniartii might have been successfully introduced from France in the 1890s.

Hegedus and Khachatourians (1993) used the restriction enzyme Sau 3A to generate a number of small randomized DNA fragments and then used dot-blot tests to test the specificity of these fragments for B. bassiana. Several fragments, ranging in size from 1180 to 2700 bp were found to hybridize to DNA from B. bassiana but not B. brongniartii, B. caledonica, B. densa, M. anisopliae, and several other species of entomopathogenic and non-entomopathogenic fungi. These results were extended in a later paper (Hegedus and Khachatourians, 1996) in which the use of combinations of three primers (P1, P3 and P5) as species-specific probes was described. The PCR products derived from the P1–P3 primer set were then studied using single-strand conformation analysis. This enabled identification of a specific strain of B. bassiana.

Polymorphism of the ITS region as detected using RFLPs was also used by Neuvéglise et al. (1994) to compare isolates of B. brongniartii from Haplochelus marginalis. This study, while confirming the high degree of variability within the ITS region of B. brongniartii, found a remarkable degree of congruence with isolates from H. marginalis having similar RFLP patterns and being the only isolates to be virulent for this scarab host. An unrooted tree computed by the PAUP program (Swofford, 1993) shows that this group of B. brongniartii from H. marginalis forms a distinct cluster, closely related to other B. brongniartii, but more distantly related from clusters containing isolates of B. brongniartii from Melolontha melolontha and isolates of B. bassiana. A complete sequence for the ITS and 5.8S RNA coding gene for two isolates of B. bassiana has recently been published by Shih et al. (1995).

The first report of a group 1 intron in an entomopathogenic fungus was by Neuvéglise and Brygoo (1994) when they found three insertion elements each 350–450 bp in the 28S ribosomal RNA gene of B. brongniartii. In a further study of 37 isolates of B. brongniartii, two isolates of B. bassiana and one isolate of Metarhizium anisopliae, Neuvéglise et al. (1997) reported 14 variant forms of introns in four different positions. Isolates from H.
marginalis all showed one of three patterns, another pattern was unique to isolates from *M. melolontha* and eight further patterns were from isolates from other hosts including the *M. anisopliae* isolate and the two *B. bassiana* isolates. Interestingly with plant parasitic fungi, similar introns have been found in the host and the fungus (Nishida and Sugiyama, 1995) suggesting that there may have been intron transfer during evolution from host to pathogen in the *Haplochelus–B. brongniartii* model.

Another promising method for genetic fingerprinting of strains is the use of teleomeric polymorphisms (Viaud et al., 1996). The teleometric sequence was cloned from another fungus, *Botrytis cinerea* and used to analyse nine isolates of *B. bassiana*. RFLP banding patterns of the amplified DNA showed that most French isolates from the European corn borer were similar, and those from other insects gave distinct fingerprints. Glare has confirmed that teleometric fingerprinting is a useful tool for comparing isolates of *Beauveria* spp (T.R. Glare, Christchurch, 1997, personal communication). Viaud et al. (1996) found that chromosome number varied between seven and eight among these isolates and that there were significant chromosomal length polymorphisms within *B. bassiana*.

In conclusion, recent PCR studies have shown:

1. That there is considerable genetic diversity within the two main species, *B. bassiana* and *B. brongniartii* and that various methods such as RAPD (Bidochka et al., 1993; Glare and Inwood, 1997; Maurer et al., 1997), teleomeric fingerprinting (Vande et al., 1996) and RFLPs (Neuvéglise et al., 1994; Glare and Inwood, 1997) are useful for distinguishing between isolates;
2. That the morphological characters are unreliable (St Leger et al., 1992a) and in particular the distinction between *B. bassiana* and *B. brongniartii* is not supported. However, it could be that a distinction should be made between ‘true’ *B. brongniartii* from strains of *B. bassiana* which tended to form more cylindrical conidia (T.R. Glare, Christchurch, 1997, personal communication);
3. That phylogenetic relationships are most reliably determined by RFLP studies using the ITS region (Neuvéglise et al., 1994; Glare and Inwood, 1997). However, this work now needs to be extended to sequence data from this region. Limited results to date suggest that *B. bassiana* and *B. brongniartii* are closely related and that four other species are distinct but correctly placed within the *Beauveria* genus. *B. bassiana*-specific gene probes have been developed (Hegedus and Khatchatourians, 1996) but have not yet been tested by other workers. Another species, *Microhilum oncoperae* is very closely related to *B. bassiana* and its taxonomic position needs to be reassessed (Ribà et al., 1994);
4. That there is no geographical clustering within the *B. bassiana–B. brongniartii* complex other than suggestion of a distinct genotype of *B. bassiana* found only in New Zealand (Glare and Inwood, 1997);
5. That there is evidence of co-evolved host-associated genotypes, shown previously for isolates from coffee berry borer (Bridge et al., 1990), with the scarabs *Haplochelus marginalis* (Neuvéglise et al., 1994), and *Melolontha melolontha*, the Sitona weevils and the pyralid, *Ostrinia nubilalis* (Maurer et al., 1997);

6. That isolates of *Beauveria* may contain group 1 introns which can be used to distinguish between isolates (Neuvéglise et al., 1994, 1997).

### 8.5 A Case Study: Variation in *Metarhizium* spp.

#### 8.5.1 Introduction

‘Green muscardine disease’ is one of the most frequently found fungal diseases of insects and occurs worldwide. The causative agent is a Hyphomycete fungus which produces uninuclear, haploid, phialidic conidia in chains. These conidia are usually green and cover the cadaver, hence the ‘green muscardine’ appearance. In nature, the fungus is found only on infected insects or as dormant conidia in the soil but, in the laboratory, it is easily grown on a wide range of media. No sexual stage is known, however some genetic exchange is possible by means of a parasexual cycle (Al Aidroos, 1980).

The genus *Metarhizium* was erected to cover all isolates of green muscardine fungus and the first species was *M. anisopliae* from Russia. It was named after its scarab host – *Anisoplia austriaca* (Zimmermann, 1993). Since then various new names have been erected for other strains which differed from *M. anisopliae* often in minor ways such as colour of the conidia. The currently accepted taxonomy owes much to papers by Tulloch (1976) and Rombach et al. (1987). Three species are recognized, distinguished by the morphology of the conidia, the conidiophores and the growth characteristics. *M. anisopliae* has green, elongate and often slightly waisted conidia produced on parallel-sided conidiophores. There are two varieties, var. *anisopliae* with smaller conidia 5–7 × 2–3 μm, and var. *majus* with conidia approximately twice that size (it has been suggested that these conidia are diploids; Samuels et al., 1989). *M. flavoviride* is slower-growing, lighter green in colour and has swollen conidia borne on swollen conidiophores. Again two varieties are recognized, var. *flavoviride* with larger conidia 7–9 × 4.5–5.5 μm, and var. *minus* with conidia 4.5–7.0 × 2–3 μm. The third species, *M. album*, has almost white conidia and grows on plates on a subhymenial zone of inflated hyphal bodies. The conidia are swollen and measure 3–6 × 1.5–2.5 μm.

Besides these three species, isolates of which are available from many culture collections, four additional species of *Metarhizium* have been described from China: *M. pingshaeme* Chen and Guo, *M. cylindrosporae* Chen and Guo, *M. guizhousense* Chen and Guo (Guo et al., 1986), and *M. taii* Liang and Liu, and its teleomorph *Cordeyceps taii* (Liang et al., 1991).
Since the publication of the Rombach et al. (1987) key, our knowledge of diversity within *Metarhizium* has increased greatly due to new strains being found and the use of biochemical and molecular techniques to provide new taxonomic characters. Morphological studies have shown that the distinction between *M. anisopliae* with cylindrical phialides and conidia and *M. flavoviride* with its swollen phialides and ovoid conidia is difficult to apply, and some isolates can display both forms depending on factors such as cultural conditions and age of culture (Glare et al., 1996). In addition, detailed measurements of conidia have failed to show a distinction between these two species (T.R. Glare, Christchurch, 1997, personal communication). Various authors have used RAPDs to establish differences between strains from sugar cane insects (Fegan et al., 1993) from cercopids and the soil in Brazil (Tigano-Milani et al., 1995c; Fungaro et al., 1996), from different countries (Leal et al., 1994a), from acridid compared with other hosts (Cobb and Clarkson, 1993; Bridge et al., 1993; Bidochka et al., 1993), and from New Zealand (Glare et al., 1997). Similar studies by RFLPs on rDNA have also shown high levels of diversity between isolates (Pipe et al., 1995). Using allozymes combined with a number of other characters, St Leger et al. (1992b) detected ‘cryptic’ species within *M. anisopliae*. Rath et al. (1995) using germination temperature profiles (McCammon and Rath, 1994) and carbohydrate utilization patterns described a new cold-temperature variety, *M. anisopliae* var. *frigidum*. A powerful method for identifying strains, useful for direct determination in infected insects, is the use of primers which detect polymorphisms in the *Pr1* gene and RFLP to produce scorable bands (Leal et al., 1996). Recently Bailey et al. (1996) have described introns in the large subunit of nuclear ribosomal RNA genes from *M. anisopliae*.

The present case study aims to extend that of Curran et al. (1994) to help resolve some of the problems with the current taxonomy of *Metarhizium*. These problems include:

1. Should the three specific names, *M. anisopliae*, *M. flavoviride* and *M. album* be retained and if so, how are they to be defined given that the morphological basis has been shown to be invalid? A radical solution, already proposed, is that *M. flavoviride* and *M. album* should be synomized with *M. anisopliae* which would then be subdivided into a number of varieties (Milner et al., 1994);

2. What is the correct name for the genetically distinct group of isolates from acridid hosts now known as *M. flavoviride* group 3 (Bridge et al., 1997)? The first of these isolates (ARSEF 2023) was isolated from an acridid in the Galapagos Islands and described primarily on the basis of conidial morphology as *M. flavoviride* var. *minus* (Rombach et al., 1986). Another isolate (ARSEF 324) was isolated from *Austracris guttulosa* from Australia and named *M. anisopliae* (St Leger et al., 1992b), again on the basis of conidial morphology. It has since been shown to be almost identical genetically to
ARSEF 2023 and a number of other so-called acridid isolates from many countries in Africa, as well as Madagascar, Brazil and Australia (Bidochka et al., 1994). Bidochka et al. (1994) revised their opinion of ARSEF 324, and on the basis of PCR-amplified banding patterns described it as *M. flavoviride*. These isolates are all very similar when studied using RAPDs, isozymes and protease production, yet are quite distinct from isolates of *M. flavoviride* from the soil or from leafhoppers in the Philippines and the Solomon Islands (Bridge et al., 1997);

3. What is the taxonomic validity of *M. anisopliae* var. *frigidum* (Rath et al., 1995)?

4. Another group of isolates, like the acridid group, which are genetically distinct from all others studied have been identified from New Zealand (Curran et al., 1994; Rakotonirany et al., 1994; Glare et al., 1997). What is the correct taxonomic placement for these isolates?

Therefore in this study, we attempt to redefine the phylogenetic, and by inference, the taxonomic relationships of the major morphological species clusters in *Metarhizium*. We correlated RAPD banding patterns with sequence data from the ITS and 5.8S rDNA. Here we report on phylogenetic estimates made by parsimony and distance using the ITS and the 5.8S region of the rDNA. The following is a summary of this work which is published in detail elsewhere (Driver et al., 1998).

**8.5.2 Methods**

**Isolates**

The 26 representative isolates used in this study are given in Table 8.1. Another 95 isolates have also been sequenced but did not provide significant additional data. The isolates were chosen either because they are the subject of on-going CSIRO research to develop a range of mycoinsecticides based on *Metarhizium* spp. (Milner and Jenkins, 1996), or because they are important (such as isolates derived from type material) for a comprehensive understanding of genetic diversity within *Metarhizium* spp.

**ITS amplification and sequencing**

PCR (Saiki et al., 1988) was used to amplify the region of the ribosomal repeat from the 3′ end of the 16S rDNA to the 5′ end of the 28S rDNA, spanning the ITS1, the 5.8S rDNA and the ITS2. Primer sequences and reaction conditions were those described by Curran et al. (1994). Sequencing reactions were done using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing mix from Applied Biosystems Inc. (ABI) Australia.

**RAPD amplifications and analysis**

The presence or absence of RAPD band patterns was scored visually in order to correlate any relationships with ITS sequence data without any attempt to produce an hierarchic arrangement of clusters. RAPD groups
Table 8.1. List of representative isolates used for case study on *Metarhizium*.

<table>
<thead>
<tr>
<th>Clade</th>
<th>Morphological species</th>
<th>FI number</th>
<th>Other designation</th>
<th>Host</th>
<th>Geographical origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>M. album</em></td>
<td>FI-MaF</td>
<td>ARSEF 1941</td>
<td><em>Nephotettii viresens</em> (Homoptera)</td>
<td>Philippines</td>
<td>Rombach et al. (1997)</td>
</tr>
<tr>
<td></td>
<td><em>M. album</em></td>
<td>FI-1165</td>
<td>ARSEF 1942</td>
<td><em>Nephotettii viresens</em> (Homoptera)</td>
<td>Philippines</td>
<td>Rombach et al. (1997)</td>
</tr>
<tr>
<td>2</td>
<td><em>M. flavoviride var. minus</em></td>
<td>FI-1173</td>
<td>ARSEF 2948</td>
<td>Unknown (Homoptera)</td>
<td>Brazil</td>
<td>Humber (1992)</td>
</tr>
<tr>
<td></td>
<td><em>M. anisopliae var. anisopliae</em></td>
<td>FI-152</td>
<td>Lepidoptera consobrina (Coleoptera)</td>
<td>Australia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>M. anisopliae var. anisopliae</em></td>
<td>FI-698</td>
<td>F10</td>
<td>Unknown (Lepidoptera)</td>
<td>New Zealand</td>
<td>Glare et al. (in press)</td>
</tr>
<tr>
<td></td>
<td><em>M. anisopliae var. frigidum</em></td>
<td>FI-1124</td>
<td>DAT-F220</td>
<td>Soil</td>
<td>Australia</td>
<td>Rath et al. (1995)</td>
</tr>
<tr>
<td></td>
<td><em>M. anisopliae var. frigidum</em></td>
<td>FI-1125</td>
<td>DAT-F368</td>
<td>Soil</td>
<td>Australia</td>
<td>Rath et al. (1995)</td>
</tr>
<tr>
<td>4</td>
<td><em>M. anisopliae var. anisopliae</em></td>
<td>FI-72</td>
<td>IMI 177416</td>
<td><em>Pemphigus treherni</em> (Homoptera)</td>
<td>Britain</td>
<td>IMI (1982)</td>
</tr>
<tr>
<td>5</td>
<td><em>M. flavoviride var. minus</em></td>
<td>FI-1172</td>
<td>ARSEF 1764</td>
<td><em>Nilaparvata lugens</em> (Homoptera)</td>
<td>Philippines</td>
<td>Rombach et al. (1986)</td>
</tr>
<tr>
<td></td>
<td><em>M. flavoviride var. minus</em></td>
<td>FI-403</td>
<td>ARSEF 2037</td>
<td><em>Nilaparvata lugens</em> (Homoptera)</td>
<td>Philippines</td>
<td>Rombach et al. (1986)</td>
</tr>
<tr>
<td>6</td>
<td><em>M. flavoviride var. flavoviride</em></td>
<td>FI-1170</td>
<td>ARSEF 2025</td>
<td>Soil</td>
<td>Germany</td>
<td>Gams and Roszypal (1973)</td>
</tr>
<tr>
<td></td>
<td><em>M. flavoviride var. flavoviride</em></td>
<td>FI-405</td>
<td>ARSEF 1184</td>
<td><em>Otiorynchus sulcatus</em> (Coleoptera)</td>
<td>France</td>
<td>Rombach et al. (1986)</td>
</tr>
<tr>
<td></td>
<td><em>M. flavoviride var. flavoviride</em></td>
<td>FI-402</td>
<td>ARSEF 2024</td>
<td><em>Otiorynchus sulcatus</em> (Coleoptera)</td>
<td>France</td>
<td>Rombach et al. (1986)</td>
</tr>
<tr>
<td></td>
<td><em>M. anisopliae var. frigidum</em></td>
<td>FI-38</td>
<td>DAT-F001</td>
<td><em>Adoryphorus couloni</em> (Coleoptera)</td>
<td>Australia</td>
<td>Yip et al. (1992)</td>
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<tr>
<td></td>
<td>M. flavoviride var. minus</td>
<td>FI-1216</td>
<td>ARSEF 2023</td>
<td>Acridid (Orthoptera)</td>
<td>Galapagos Islands Rombach et al. (1986)</td>
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<tr>
<td>7</td>
<td>M. anisopliae var. anisopliae</td>
<td>FI-985</td>
<td>ARSEF 324</td>
<td>Austracris guttulosa (Orthoptera)</td>
<td>Australia St Leger et al. (1992)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M. flavoviride</td>
<td>FI-987</td>
<td>IMI 330189</td>
<td>Ornithacris cavoisi (Orthoptera)</td>
<td>Niger Bridge et al. (1993)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. flavoviride</td>
<td>FI-1028</td>
<td>IMI 324673</td>
<td>Zonocerus variegatus (Orthoptera)</td>
<td>Tanzania Bridge et al. (1993)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>M. anisopliae var. anisopliae</td>
<td>FI-1042</td>
<td>Lepidota consobrina (Coleoptera)</td>
<td>Australia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. anisopliae var. anisopliae</td>
<td>FI-1029</td>
<td>IMI 168777</td>
<td>Schistocerca gregaria (Orthoptera)</td>
<td>Eritrea Tulloch (1976)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. anisopliae var. anisopliae</td>
<td>FI-1034</td>
<td>IIBC I91-614</td>
<td>Patanga succincta (Orthoptera)</td>
<td>Thailand Bidochka et al. (1994)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. anisopliae var. anisopliae</td>
<td>FI-1099</td>
<td>ARSEF 445</td>
<td>Teleogryllus commodus (Orthoptera)</td>
<td>Australia Bridge et al. (1993)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M. anisopliae var. majus</td>
<td>FI-388</td>
<td>ARSEF 1914</td>
<td>Oryctes rhinoceros (Coleoptera)</td>
<td>Philippines Humber (1992)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. anisopliae var. majus</td>
<td>FI-389</td>
<td>ARSEF 2151</td>
<td>Oryctes rhinoceros (Coleoptera)</td>
<td>Indonesia Humber (1992)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Beauveria bassiana</td>
<td>FI-297</td>
<td>Soil</td>
<td>Australia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Paecilomyces sp.</td>
<td>FI-360</td>
<td>Inopus rubriceps (Diptera)</td>
<td>Australia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Paecilomyces sp.</td>
<td>FI-442</td>
<td>Inopus rubriceps (Diptera)</td>
<td>Australia</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Outgroups.*
were assigned on the basis of perfectly identical, or nearly identical, banding patterns (Fig. 8.1).

Analysis of rDNA sequences
Sequences for complementary strands were checked and the resulting sequences aligned using CLUSTAL W (T. Gibson, D. Higgins, J. Thompson, EMBL, Heidelberg, Germany, May 1994) at default settings. The alignment was checked visually and minor adjustments made manually. The alignment

![Fig. 8.1. RAPD–PCR banding patterns using two primers, OP-H01 and OP-F06. Panels A and B, lanes 1–17: 100 bp ladder; FI-1216 (ARSEF 2023, clade 7); FI-152, 1173 (clade 2); FI-403, 1172 (clade 5); FI-405, 402, 1170, 38 (clade 4); FI-72, 1101 (clade 6); fi-698, 699, 702, 1125, 1126 (clade 2); and panel B only FI-1165 (M. album, clade 1). Panels B and C illustrate the genetic homogeneity of acridid strains (clade 7), lanes 1–15: 100 bp ladder; FI-1216 (Galapagos Islands); FI-1189, 1190, 1191, 1192, 1193 (Brazil); FI-1067, 983, 984, 986 (Benin); FI-987 (Niger); FI-1028 (Tanzania); and FI-985 (ARSEF 324), 1155 (Australia).]
was translated into #NEXUS format for analysis using PAUP4d52-53 (beta test version of PAUP; Swofford, 1997).

The partition homogeneity test (Farris et al., 1995) as implemented in PAUP indicated no incongruence amongst the ITS1, 5.8S and ITS2 regions of the molecule. Subsequently the regions were analysed separately and together, but final phylogenetic results are based on the combined data. The ITS1 region (positions 14–219 in the file) contained 71 parsimony informative positions, the 5.8S region (positions 220–377) contained seven, and the ITS2 region (positions 378–604) contained 80. With alignment gaps treated as missing data and with no differential weighting of transversions against transitions, parsimony analysis of either the whole data set or the ITS1 region identified in excess of 40,000 most parsimonious (mp) trees, apparently on a single TBR-branch-swapper defined island. Separate analysis of the ITS2 region produced a lesser number of mp trees, the strict consensus of which was consistent with the strict consensus of the ITS1 or whole data set trees (Fig. 8.2). Some branches are supported by several synapomorphic changes. The large number of trees results from the lack of informative character state changes within certain clades (primarily single point deletions in a number of M. anisopliae isolates).

8.5.3 Results

The RAPD–PCR banding patterns showed a high level of diversity even within some clades, for example clade 9 which contains a very large number of isolates normally classified as M. anisopliae var. anisopliae. Small changes in ITS sequence, e.g. single point mutations or deletions with respect to the type strain of M. anisopliae var. anisopliae, are often accompanied by dramatically different RAPD patterns with most primers. Low levels of sequence divergence in most other clades of isolates, including var. majus and the acridid isolates, were mirrored by much smaller levels of polymorphism in their RAPD banding patterns (Fig. 8.1, panels A, B, C and D).

The size of the ITS1, 5.8S and ITS2 PCR product (excluding primer sequences) varied from 506 bp for FI-1029, the type material of M. anisopliae var. anisopliae, to 573–4 bp for the two isolates of M. album. The size of this region is comparable to that reported for other entomopathogenic fungi (Neuvéglise et al., 1994; Anderson et al., 1997). Size polymorphisms for this region which could be detected on agarose gels, were most evident between isolates from var. anisopliae, var. majus and B-type strains of Metarhizium, clades 8, 9 and 10 which ranged from 505 to 510 bp, compared with all other material examined, including the acridid isolates. These larger ITS fragments, 542–544 bp in acridid isolates, 530–551 bp for all varieties of M. flavoviride, 545–546 bp for New Zealand isolates and 552–558 bp for the two isolates from clade 2 in the phylogenetic tree, generally arose from longer, more variable sequences in the ITS1. Interisolate variation was very low in all recognized varieties of Metarhizium, and newly identified clusters with most
Fig. 8.2. Phylogeny of *Metarhizium* spp.: strict consensus of a maximum parsimony analysis. The numbers at branches are the bootstrap values (100 replicates), and *** indicates a T-PTP value of 0.01. Isolate designations are given in Table 8.1.
isolates exhibiting only single point mutations, or small insertions or deletions, which were generally confined to ‘hot spots’ of variation.

The strict consensus tree of length 394 steps from the whole data set, original alignment, with uncorrected distances, is shown as Fig. 8.2. Bootstrap tests (Felsenstein, 1985) were used to quantify the support for nodes in the consensus of mp trees. Taxon groups distinguished only by parsimony-uninformative state changes (i.e. isolates differing by only single point deletions) were combined into single nodes. Bootstrap analysis was based on 100 bootstrap replicates each with ten random-addition-sequence starting trees and keeping no more than 50 trees at each replicate, to sample the range of parsimonious trees for each resampling of the data matrix. Bootstrap proportions > 50% are shown in Fig. 8.2 where the monopoly of clades 7–10 (M. anisopliae) is supported.

Topologically constrained permutation tail probability (T-PTP) tests (Faith, 1991) under both equal-weighting and 2:1 transversion:transition weighting confirmed the apparent monopoly of the M. anisopliae clades and clades 4 and 5 (M. flavoviride). These clusters are not due to chance alone (PTP = 0.01). The acridoid isolates, FI-985, FI-986 and FI-1028, appear as a clade 7 closely related to clades 9 and 10 which are typical M. anisopliae at bootstrap support > 95%, rendering M. flavoviride paraphyletic. T-PTP was used to test this clustering against a conflicting hypothesis that the acridid isolates should cluster within a monophyletic clade of M. flavoviride. For this test, the M. anisopliae clades were reduced to their basal node and the group M. anisopliae + clade 7 isolates subjected to T-PTP tests both for monophyly and non-monophyly. A prior hypothesis of monophyly could not be falsified (PTP = 0.01). A prior hypothesis of non-monophyly could be falsified (PTP = 1.00). The data are sufficient to accept the former hypothesis and reject the latter.

Clade 6 includes isolate FI-38 which is now a commercial product in Australia (BioGreen™) under the name M. anisopliae, clusters within M. flavoviride var. flavoviride on all mp trees. That placement is supported by bootstrap values > 80% on the intervening branches. To confirm the prior hypothesis, a placement within the M. anisopliae clade could be falsified on these data, and T-PTPs for monophyly of clade 4 + M. anisopliae isolates were conducted. A prior hypothesis of monophyly could be falsified (PTP = 1.00). A prior hypothesis of non-monophyly could not be falsified (PTP = 0.01). These tests confirm placement of FI-38 within clade 6 (M. flavoviride).

The ITS region has been used extensively for fungal taxonomy (reviewed by Seifert et al., 1995), and it is very useful for identifying species clusters, as well as differentiating strains that are ecologically distinct (Nazir et al., 1991). Sequence data from this region (Driver et al., 1998), have provided a sound framework for a taxonomic revision of the three accepted species of Metarhizium, despite the lack of resolution in the base of the tree. Processes such as gene conversion may result in the loss of clear patterns of descent in
distant relationships, and reticulation and not synapomorphies arise in the comparisons of sequences (Brower and DeSalle, 1994). The results show that the worldwide genetic diversity, as presently known, can be circumscribed by ten clades. In most cases these clades correspond to either morphologically recognized varieties, or clusters of isolates which have been identified by other molecular or biochemical markers. It is likely that most new isolates found in the future will be unequivocally placed in one of these clades. However the taxonomy is flexible and new clades may be added if the sequence data justify creation of additional clades. A key question is what level of nucleotide divergence should be attributed to define taxonomic rank at species and varietal levels? Nucleotide divergence for the ITS region between morphologically defined species in *Metarhizium* ranges between 14 and 18%, whilst divergence between recognized varieties within species does not exceed 5%. Levels of nucleotide divergence for clusters of isolates from acridoid hosts in New Zealand blur the margins between species and varietal limits. Paired clusters of isolates of clade 7/ *anisopliae*, clade 7/ *flavoviride* and clade 7/ *album* show levels of nucleotide divergence of 9.4%, 17.2% and 16.5% respectively, whilst paired clusters of isolates from clade 3/ *anisopliae*, clade 3/ *flavoviride*, and clade 3/ *album* are in the range 11.3%, 6.9% and 9.1% respectively. Cladistics requires that species represent monophyletic groups (Wiley et al., 1991) but there are no guidelines on taxonomic ranking (Seifert et al., 1995).

As far as possible, it is proposed that existing species names be retained and any new names proposed will be at the variety level. Therefore the name *M. album* is retained and is now defined as applying only to isolates which fit into clade 1. At present we consider clades 2–6 to contain varieties of *M. flavoviride* while clades 7–10 contain varieties of *M. anisopliae*. Where appropriate, new varietal names have been proposed for some of these clades (Driver et al., 1998). However for some clades, we have too few isolates and therefore too little data, on genetic diversity within the clade, to justify a varietal name at this time. It is likely that as our knowledge of these clades increases, especially with regard to ecological factors such as host specialization, the taxonomy will become clearer, leading to some clades being elevated to new species.

### 8.6 Descriptions of the Clades

Most taxonomic decisions are implicitly based on the biological species concept of actually or potentially interbreeding populations that are reproductively isolated, a view that has been widely accepted by mycologists (Seifert et al., 1995). It has been suggested that anamorphic species not be considered as species at all, and that the term be reserved for organisms that undergo meiosis (Perkins, 1991). In practice, morphologically defined species infer the potential to interbreed (Volger and Desalle, 1994). Such
biological species concepts of actual or inferred breeding populations to delimit taxa are not directly applicable to *Metarhizium*. The molecular data give qualified support for the existing morphological-based taxonomy, if we accept that species represent monophyletic groups. The combination of molecular, biochemical and morphological markers make a case for phylogenetically defined species or ‘evolutionary significant’ groups (Volger and Desalle, 1994).

**Clade 1 – *Metarhizium album***
The only isolates identified as belonging to this clade were isolates ARSEF 1941 and ARSEF 1942, both of which were described by Rombach *et al.* (1987) from infected leafhoppers in the Philippines. Besides the apparent host specificity of this clade, other characters are the small ovoid to ellipsoid conidia measuring 4–6 × 1.5–2.5 µm, the growth of a bulging mass of hyphal bodies rather than mycelium prior to sporulation and the lack of laterally adhering conidia forming prismatic columns. The pale brown colour of the spores is also characteristic although this is of doubtful taxonomic significance.

**Clade 2 – *Metarhizium flavoviride***
This is an unusual clade containing just two rather diverse isolates. The first, FI-1173 (ARSEF 2948) on an homopteran from Brazil was identified as *M. flavoviride* var. *minus* by Humber (1992), however all the other isolates of this variety from leafhoppers and related insects in the Philippines and Solomon Islands (Rombach *et al.*, 1986) are clustered in clade 5. Morphologically these isolates are all very similar and come from similar hosts, thus it is surprising that one of the isolates should be genetically so distinct. Even more surprising is that FI-152, isolated from a scarab in Australia, and morphologically resembling *M. anisopliae* var. *anisopliae*, should also cluster into this clade. FI-152 and FI-1173 show RAPD–PCR patterns which clearly distinguish them from other isolates of *M. flavoviride* var. *minus*. More work is needed to clarify this clade and to determine if there are ecological and/or morphological characteristics which are shared by these isolates, and to collect other isolates from nature which might fit into this clade.

**Clade 3 – *Metarhizium flavoviride***
This clade contains quite a large number of isolates, many from New Zealand, which have been recognized as distinct by several workers (Riba *et al.*, 1990; Curran *et al.*, 1994; Glare *et al.*, 1997). Our study has shown that isolates from this clade also occur in Australia (described as Strain 2 by Yip *et al.*, 1992) and that they have been included as part of a group of low-temperature isolates called *M. anisopliae* var. *frigidum* by Rath *et al.* (1995). These authors recognized that some isolates were as distinct from *M. anisopliae* var. *anisopliae* as they were from *M. flavoviride* and that they may
represent a new species. Morphologically, isolates from this clade have cylindrical conidia and correspond to the short-spored form of *M. anisopliae* described from New Zealand (Glare *et al*., 1997). All isolates in this clade grow well at low temperatures (10°C and below), infect soil insects or have been isolated from the soil and attack soil insects (both scarab larvae and lepidopterous caterpillars).

**Clade 4 – *Metarhizium flavoviride***

This is another small clade containing just two isolates with identical collection data except the date. Both isolates were from infected root aphids in Norfolk, UK (Foster, 1975), and they have cylindrical green conidia morphologically resembling *M. anisopliae* var. *anisopliae*. However they grow well at low temperatures and cluster with *M. flavoviride*; consequently, we regard that as the correct specific name. More isolates representing this clade are needed before it can be formally given a varietal name and it is possible that a search for *Metarhizium* infection on other root aphids may reveal additional isolates.

**Clade 5 – *Metarhizium flavoviride* var. *minus***

In our study only two isolates FI-403 and FI-1172 (ARSEF 2037 and ARSEF 1764), both morphologically identified as *Metarhizium flavoviride* var. *minus* and obtained from infected leafhoppers, were included within this clade. One of these, FI-403 (ARSEF 2037) was identified by Rombach *et al.* (1985) as the type-isolate of this variety. These isolates fit the description given by Rombach and were collected in the Philippines and the Solomon Islands suggesting a narrow host range and geographical distribution.

**Clade 6 – *Metarhizium flavoviride* var. *flavoviride***

This clade contains FI-405 (ARSEF 2025) which was derived from the material used by Gams and Roszypal (1973) for their original description of *M. flavoviride*. It also contains other isolates described by Rombach *et al.* (1986) as *M. flavoviride* var. *flavoviride*. These isolates have large, somewhat swollen conidia which form a pale green conidial mat in culture and come from the soil or from soil-inhabiting beetles. All these isolates grow well at low temperatures. Rath *et al.* (1995) included FI-38 in *M. anisopliae* var. *frigidum* and this isolate, along with a number of other soil-derived isolates, has an ITS sequence similar to FI-405 (ARSEF 2025), unequivocally identifying these isolates as *M. flavoviride* var. *flavoviride*. RAPD–PCR banding patterns show a high degree of homogeneity and conservation with the northern European isolates suggesting that this clade probably has a very wide geographical distribution. Rath *et al.* (1995) provide a nearest-neighbour dendrogram based on their carbohydrate data which showed that FI-38 (DAT F001) clustered nearer to *M. flavoviride* than to *M. anisopliae*. Our data support this finding and it is therefore proposed that the correct identity of this isolate is *M. flavoviride* var. *flavoviride*. 
Clade 7 – *Metarhizium anisopliae*

This clade contains all of the acridoid isolates described as ‘*M. flavoviride* group 3’ (Bridge et al., 1997). Our data supports and extends that of Bridge et al. (1993), Cobb and Clarkson (1993), Bidochka et al. (1993) and Bridge et al. (1997), in showing that these isolates are genetically quite uniform and are quite distinct from the type material of both accepted varieties of *M. flavoviride*. Bootstrapping and T-PTP tests give strong support for the phylogenetic signal that unites these isolates to the major evolutionary line that gives rise to *M. anisopliae* rather than *M. flavoviride*. Therefore, we propose that the correct identity is as a new variety of *M. anisopliae* which will be described elsewhere (F. Driver, unpublished work).

The isolates in this clade have all been isolated from acridid grass-hoppers. Interestingly other orthopteran hosts such as crickets are not susceptible and in nature are attacked (exclusively) by isolates from clade 9. In our experience, grasshoppers are more often infected in nature by isolates from clade 9 but these isolates are less virulent than the rarer clade 7 isolates (Bateman et al., 1996).

Most isolates from clade 7 produce small ovoid conidia which would identify them as *M. flavoviride* var. *minus*. For example, FI-1216 (ARSEF 2023) from the Galapagos Islands described under that name by Rombach et al. (1985). Other isolates, though, have larger spores which can be almost cylindrical (FI-985, ARSEF 324). All isolates share some unusual characteristics such as the ability to sporulate internally and to grow well at temperatures up to 40°C (Welling et al., 1994).

Clade 8 – *Metarhizium anisopliae*

This is another clade with only a small number of known isolates. All known isolates have cylindrical spores and produce a profuse layer of green conidia in culture, and have been isolated from scarab larvae in Queensland, Australia. Using allozyme data St Leger et al. (1992b) were able to show considerable inter-isolate variation and the existence of cryptic ‘species’ or, probably more correctly, cryptic varieties within *M. anisopliae*. This cluster of isolates has previously been described as B-type isolates of *M. anisopliae* (Curran et al., 1994), and probably correspond to those of pathogenicity group 1 for *Lepidiota* spp; or RAPD group A, described by Fegan et al. (1993). More needs to be known about this clade; however, the sequence data clearly separates it from clades 7 and 9.

Clade 9 – *Metarhizium anisopliae* var. *anisopliae*

The type material of *Metarhizium anisopliae* var. *anisopliae* as described by Tulloch (1976) is FI-1029 (IMI168777ii) and this isolate therefore forms the basis for this clade. This clade includes the vast majority of isolates found in nature and is genetically highly diverse. St Leger et al. (1992b) demonstrated the existence of clonal population structures in *M. anisopliae*. Distinct groups can be identified within the clade: for example, a number
of isolates from the black field cricket, *Teleogryllus commodus*, in Australia, share identical ITS sequence data and have very similar RAPD patterns (Milner *et al*., 1996). Isolates generally have cylindrical conidia, 5–7 µm long which form green conidia in columns of chains. They normally grow poorly outside the range 15–32°C, although recently two cold temperature active isolates have been found in soil samples taken at MacQuarie Island (Roddam and Rath, 1997), confirming that cold activity is a homoplasious character.

**Clade 10 – Metarhizium anisopliae var. majus**

A typical isolate from this clade is FI-401 (ARSEF 1946) which conforms to the description by Tulloch (1976). Isolates are readily identified on the basis of the very large conidia, usually over 10 µm long, and rapidly growing colonies producing dark green conidia; they are most frequently found attacking dynastine beetles in tropical countries. Our results support those of other workers (St Leger *et al*., 1992b; Leal *et al*., 1994b) showing that genetically this clade differs less from *M. anisopliae* var. *anisopliae* isolates in clade 9 than do other isolates such as those in clade 8 (B-type), which are also currently described as var. *anisopliae*.

### 8.7 Discussion

Morphological characters are generally complex and may involve the expression of many genes. Traditional fungal systematics assesses the phenotype for reliable characters that should result in the recognition of homologous features with alternate character states that may be evaluated cladistically (Brower and Desalle, 1994; Seifert *et al*., 1995). The skill of the researcher resides in the ability to ‘assign characters successfully to the appropriate level of analysis’ (Brower and Desalle, 1994). The reproducibility and impartiality of sequence-based systematics has made an important contribution to fungal taxonomy. Although species concepts based solely on a single gene region have been considered insufficient and too trivial to be representative of all characters (Seifert *et al*., 1995), gene regions such as the ITS are being heavily relied upon to discriminate and define new species in such genera as *Ascosphaera* (Anderson and Gibson, 1997), and the edible shiitake mushroom *Lentinula* (Hibbert *et al*., 1995), as well as phytopathogenic fungi such as *Colletotrichum* (Sherriff *et al*., 1994). 18S ribosomal DNA sequence data have been used to estimate ascomycete relationships, and phylogenetic trees from such data sets are highly resolved, and generally consistent with morphological evolutionary events like forcible spore discharge (Berbee and Taylor, 1995). Lutzoni and Vilgalys (1995) integrated molecular and morphological data sets to estimate fungal phylogenies in lichenized and non-lichenized *Omphalina* species. Homogeneity testing of the 28S large subunit ribosomal DNA sequences and the morphological
characters showed that the two data sets were sampling the same phylogenetic history and could be combined.

Such studies go to the heart of the matter, that is, the conflict of gene trees and the evolution of a segment of DNA and the ‘true’ course of evolution of the organism. Brower and Desalle (1994) consider such concerns about gene tree versus species tree problems to be overstated, and except for recently diverged or hybridizing taxa, ancestral polymorphism and lineage sorting cannot result in strong support for an incongruent topology. Congruence between cladograms derived from different gene regions or other available characters into a total evidence approach such as described above provides strong statistical support and circumvents the problem. The gene region of choice is critically important in maximizing the phylogenetic signal-to-noise ratio. Reviews by Simon et al. (1994) on the utility of mitochondrial gene sequences, and Brower and DeSalle (1994) on using nuclear gene regions, raise important issues about the taxonomic rank at which particular genes are useful.

8.7.1 How can the taxonomy be made more user friendly?

A major problem with a taxonomic scheme which is dependent to a large extent on molecular data is that it is difficult to provide easily applied diagnostic characters for identification. While some clades will be easy to identify others, such as clade 2, are impossible at the present time except by molecular methods. Published molecular methods for species recognition such as the probes devised by Bidochka et al. (1994) and Leal et al. (1994b) cannot be applied since they did not include all available type material, consequently there is uncertainty about the true identity of the isolates which they used as, for example, *M. flavoviride* var. *flavoviride*. Only the Gams and Roszypal (1973) strain, ARSEF 2025, can be regarded as *M. flavoviride* var. *flavoviride* sensu stricto. Consequently these methods need to be reassessed in the light of the results of our study.

While our work suggests that the most rigorous way to identify clades is by sequence determination of the ITS regions, it has also been shown that these correlate strongly with RAPD patterns, in the same manner that ITS-RFLP patterns have been shown to correlate with RAPD patterns in *Beauveria* (Maurer et al., 1997). It is therefore suggested that clades be identified by using either RFLP analysis of the ITS region, or RAPD–PCR patterns established by primers such as OP-H01 or OP-A03 which give rise to fewer polymorphic fragments against a set of ‘standard’ isolates. It is suggested that the first isolate listed under each clade in Table 8.1 be used as the standards or type material. These are mostly ARSEF isolates and it is hoped that the other isolates will be deposited prior to publication of the taxonomic revision (Driver et al., 1998).
8.8 Conclusions and Future Work

Molecular methods involving PCR techniques are now accepted as being a key element in research on entomogenous fungi and some information, as summarized in this chapter, is available about most groups. However, it is still an infant technology which means that there are still substantial gaps in our knowledge: for example, clarification of strain variation in the *Entomophthora muscae* complex, a common worldwide pathogen of flies, is needed and may shed light on differences between countries in the ecology of these pathogens. In addition, there are new techniques, and improvements to existing techniques, which have yet to be applied to entomogenous fungi. Taxonomic problems, such as those highlighted in the case study on *Metarhizium* spp., could be further resolved by an analysis of mitochondrial genes (as initiated by Junior and Martinez-Rossi, 1995) or nuclear protein coding genes such as the Pr1 protease gene used by Leal et al. (1996) for strain identification. Other techniques which have either not been applied or only used to a limited extent include random amplified microsatellites (RAMS; Hantula and Muller, 1997) and amplified fragment length polymorphisms (AFLP; Majer et al., 1996). The presence of double-stranded RNA and isometric virus-like particles in *M. anisopliae* was first announced by Leal et al. (1994a) and has recently been confirmed by Bogo et al. (1996). Similar viruses may be present also in *Beauveria bassiana* (Osborne and Rhodes, 1994). However, their possible significance in terms of virulence is unknown. In fact, very little is known of virulence genes in entomogenous fungi, though the Pr1 gene is involved in *M. anisopliae* (St Leger et al., 1996) and probably similar genes occur in some other entomogenous fungi (Leal et al., 1996). With the increased use of these fungi in pest control there will be more attention given to virulence factors and the development of genetically improved, hypervirulent, isolates as reported recently by St Leger et al. (1995).

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Application of PCR in Fungal Biotechnology

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9.1 Introduction

Filamentous fungi have been utilized for decades in the commercial production of enzymes, antibiotics, and speciality chemicals. Fungi are unparalleled as producers of secreted proteins; *Aspergillus niger*, for example, produces glucoamylase at 20 g l⁻¹. Since the development of transformation methods for filamentous fungi, genetic engineering technology has been applied to fungal systems for the production of heterologous proteins. Members of the genus *Aspergillus, Fusarium* and *Neurospora* in particular, have been employed as hosts for the high level secretion of recombinant proteins (Peberdy, 1994; Van den Hombergh et al., 1996). In this chapter, we will discuss the application of various PCR techniques for the characterization, cloning and expression of heterologous genes in industrially important filamentous fungi. Characterization of fungal strains and transformants, as well as the development of suitable fungal hosts for the expression of therapeutic human proteins, will also be discussed.

9.2 Cloning of Conserved Genes by PCR

Several approaches have been used to isolate genes from filamentous fungi (Goosen et al., 1992). A number of fungal genes were cloned by complementation of auxotrophic mutants of *Escherichia coli* or *Saccharomyces cerevisiae*. This approach is limited because fungal expression signals may not be recognized by the host transcriptional or translational machinery, and many fungal genes have introns which cannot be processed.
properly to make a functional mRNA in *E. coli* or yeast. Isolation of fungal genes by complementation of auxotrophic fungal mutants is limited by the low DNA transformation efficiency in filamentous fungal species with the exception of *Aspergillus nidulans* and *Neurospora crassa*. A ‘reverse genetics’ approach has been used successfully in the cloning of many fungal β-lactam biosynthetic genes (Skatrud, 1992). This approach begins with the purification of the desired enzyme, determination of a partial amino acid sequence, and synthesis of oligonucleotide probes based on the amino acid sequence. A genomic DNA library of this fungal species is then constructed in *E. coli* and screened for clones that hybridize to the DNA probes. Sequencing of the cloned DNA is used to confirm that the DNA sequence matches the known amino acid sequence. For genes which are sufficiently conserved, gene isolation can be achieved in a short period of time by heterologous hybridization with a probe generated from its counterpart gene from another organism. Alternatively, degenerate oligonucleotide primers synthesized from the conserved regions of the available genes, are used to amplify a genomic or a cDNA fragment that can be used as a probe for cloning of the entire gene. This approach has been used successfully for the cloning of the cDNA of the NADPH-cytochrome P450 reductase gene (*cpr*) from *Schizosaccharomyces pombe* (Miles, 1992) as well as the genomic *cpr* gene from *A. niger* (Van den Brink *et al.*, 1995), and the cDNA clone of the protein disulphide isomerase gene from *A. niger* (Malpricht *et al.*, 1996).

### 9.2.1 Cloning of a gene from genomic DNA

The NADPH-cytochrome P450 reductase (CPR) serves as an electron donor for the activity of all eukaryotic non-mitochondrial cytochrome P450 monooxygenase enzymes. These enzymes catalyse the oxidation of lipophilic chemicals by the addition of one atom of molecular oxygen to the substrate molecule, such as the conversion of benzoic acid to *p*-hydroxy-benzoic acid. Genomic and cDNA clones of the *cpr* gene have been isolated from yeast, rat, rabbit and human genomes. The amino acid sequence of trout and pig CPR have also been determined from the purified protein. Comparison of the deduced amino acid sequences of these proteins shows two highly conserved regions. Miles (1992) used degenerate oligonucleotide primers, based on these conserved sequences, to amplify a 309 bp fragment of the *cpr* gene from *Sc. pombe* and used it as a probe to identify a cDNA clone of the *cpr* gene. Van den Brink *et al.* (1995) used a similar approach to generate a 1.4 kbp PCR-amplified DNA probe and cloned a genomic fragment containing the entire coding region with the 5′ promoter and 3′ untranslated regions of the *cpr* gene from *A. niger*. This method should be generally applicable to the cloning of *cpr* genes from diverse sources when hybridization with heterologous probes fails.
9.2.2 Cloning of a gene from mRNA

Protein disulphide isomerase (PDI) catalyses the formation, reduction, and isomerization of disulphide bonds in the process of maturation and folding of nascent polypeptide chains in the cell. Comparison of amino acid sequences of PDI proteins from a number of organisms indicates that the PDI enzyme has two highly conserved active sites. Malpricht et al. (1996) cloned the PDI cDNA sequences from total RNA isolated from mycelia of *A. niger* NRRL3 in three steps. First, they used two degenerate primers derived from the conserved regions of the two active sites to amplify a 158 bp PCR fragment from single-stranded cDNA which had been transcribed from *A. niger* total RNA. This PCR fragment was cloned and sequenced to verify its identity with the published PDI protein sequences. From the sequence information, a specific 27-mer primer was used as a 5′ primer in a 3′-rapid amplification of cDNA ends (RACE) PCR with single-stranded cDNA and oligo-dT primer to yield a longer cDNA fragment. This fragment consists of the above 158 bp fragment sequence and all of the 3′ sequences extending to the poly(A)-tail (Frohman et al., 1988). The 5′ end of the gene, upstream of the sequenced region, was subsequently cloned by inverse PCR (IPCR) (Huang et al., 1990). Double-stranded cDNA was prepared from full-length mRNA and self-ligated to generate circular templates for PCR. Primers were prepared which were complementary to the 5′ and 3′ ends of the previously amplified cDNA fragment, but oriented outward so that they would amplify the uncharacterized portion of the template. After 25 cycles of PCR, a DNA fragment containing sequences from the 5′ end of the mRNA was synthesized and characterized. By using IPCR, it was possible to determine the sequence of the 5′ region of a specific mRNA molecule without knowing the sequence of the 5′ end in advance. An alternative approach is to use 5′-RACE. Single-stranded cDNA is synthesized from mRNA and C-tailed using terminal deoxynucleotidyl transferase and dCTP. This serves as a template for PCR using oligo-dG and a gene-specific primer. Either method allows full-length cDNA fragments to be generated even with limited nucleotide sequence information (Frohman et al., 1988; Zilberberg and Gurevitz, 1993).

9.3 Expression Vector Construction

Interest in the use of filamentous fungi for the production of both industrial enzymes and therapeutic proteins has increased significantly in recent years. Because fungi can produce large quantities of secreted proteins and many fungal strains are now generally recognized as safe, they can potentially serve as ideal hosts for the expression of heterologous genes. To accomplish this, a transformation protocol must be developed which allows foreign DNA to be introduced into the host in either an integrated or autonomous form.
Then, an expression vector must be constructed which provides the gene of interest with additional DNA sequences to allow its expression in the fungal host. These sequences typically include:

- a strong transcriptional promoter (preferably regulated);
- suitable restriction sites near the promoter to insert heterologous genes;
- a transcriptional terminator;
- a selectable marker.

Optionally, the vector may contain a secretion signal sequence or part of a highly expressed endogenous protein (generally from the gene which is naturally associated with the strong promoter), allowing a foreign gene to be either secreted or expressed as a fusion protein.

PCR is particularly useful in the construction of new expression vectors since most of the components can be derived from existing vectors, but require several changes in the flanking restriction sites. The following example from our laboratory demonstrates how we have easily constructed a rather complex expression system for *Fusarium oxysporum* by PCR.

In the course of developing an enzymatic method to convert penicillin V to 6-aminopenicillanic acid, research scientists at Bristol-Myers Squibb discovered a strain of *F. oxysporum* which produces substantial quantities (1–2 g l\(^{-1}\)) of a penicillin V amidase (PVA) enzyme (Fig. 9.1). Expression of the PVA gene is induced by the presence of phenoxyacetic acid (POAc), and the protein is secreted into the medium. To increase production of the enzyme, we cloned the PVA gene and approximately 2 kb of flanking sequences into a vector that contained a phleomycin resistance gene for the selection of fungal transformants. When the transforming plasmid was inserted into the chromosome of a different *F. oxysporum* strain (f. sp. *lycopersici*, ATCC 16322) which has very little endogenous PVA activity and no DNA sequences homologous to the PVA gene, the transformants produced over 1 g l\(^{-1}\) of active enzyme. When the host was transformed with a vector containing a tandem repeat (two copies) of the PVA gene, the expression increased to 10 g l\(^{-1}\) (Chiang *et al.*, 1996). The protein was secreted and its expression remained POAc-inducible. These characteristics have led us to believe that the PVA promoter and gene can be converted into a component of a valuable expression system in *Fusarium*.

Because the two-copy PVA gene vector produces much higher levels of expression, we decided to create a *Fusarium* expression system with two component plasmids for the easy construction of two-gene expression vectors. One component, pWB20 (the ‘cassette’ vector, Fig. 9.2), allows a heterologous gene to be inserted into the PVA gene at a variety of locations. Depending upon the insertion site, the gene can be expressed directly, or fused to the PVA secretion signal sequence, or expressed as a fusion protein. After the gene is inserted, an expression cassette containing the gene plus the PVA promoter and transcriptional terminator can be cut out with either of two restriction enzymes (EcoRI or XbaI). A second component, pSJC62 (the ‘resistance’
vector, Fig. 9.2), contains the gene coding for phleomycin resistance and unique EcoRI and XbaI restriction sites on opposite sides of the gene. Two copies of the expression cassette can be inserted into these sites on pSJC62 to generate four different combinations. These copies are not true tandem repeats since they are separated by the phleomycin resistance gene. In order to generate tandem repeats we engineered an overlapping dam-methylation site (GATC) at one of the XbaI sites of the pWB20 cassette vector. The vector is initially grown in a dam– E. coli host so that the cassette can be cut out with XbaI. After the first copy of the cassette is inserted into the pSJC62 resistance vector, it is grown in a dam+ E. coli host which prevents cleavage of one of the XbaI sites. A second copy of the expression cassette is now easily inserted into the one remaining XbaI site to produce a true tandem repeat.

The cassette vector, pWB20 was created from the vector pFO20, which contains the PVA gene plus 1.2 kb of upstream (promoter region) and 0.8 kb of downstream (transcriptional terminator region) DNA sequences inserted into the popular cloning vector pUC19. To convert pFO20 to pWB20, it was necessary to remove six restriction sites, add four new restriction sites (one with an overlapping dam methylation site), and to modify the translation start.

![Penicillin V to 6-AP A conversion](image)

**Fig. 9.1.** Enzymatic conversion of penicillin V to 6-aminopenicillanic acid (6-APA).

![Plasmid maps of pWB20 and pSJC62](image)

**Fig. 9.2.** Plasmid maps of pWB20 and pSJC62.
site. Although introducing this number of changes appears daunting, it was easily accomplished by PCR with carefully designed primers. The strategy is outlined in Fig. 9.3. Firstly, an NcoI site was removed from the transcriptional terminator by digestion, filling-in, and religation. Next, PCR primers were made to amplify either the promoter region or the coding and terminator regions. The primers were 29–33 bp long and their 3′ ends provided 14–19 bases of homology to the PVA gene. The 5′ sequences of the primers were designed to create all of the new restriction sites needed for pWB20. For the annealing step of the PCR, the $T_m$ is about 50°C when the template is the pFO20 vector (since only the 3′ end of the primer hybridizes). After the initial rounds of PCR, however, the newly synthesized template will be complementary to the entire primer and the $T_m$ will increase to 80–90°C. To optimize the specificity of the PCR, it was carried out in two stages. Firstly, a PCR with an annealing temperature of 48°C was run for six cycles to generate a small amount of newly synthesized template. This was followed by a two-step PCR (94° and 70°C) for 35 cycles to amplify the fragments. The PCR-generated fragments were then digested with restriction enzymes to produce ‘sticky’ ends and ligated into a modified pUC19 vector (pUC19EB) which had most of the polylinker region removed. The resulting pWB20 vector is now being developed to express heterologous genes in its *Fusarium* host.

### 9.4 Site-directed Mutagenesis by PCR

After an expression vector (usually a plasmid) has been constructed, it is often desirable to introduce mutations into the vector either to alter the properties of an expressed gene product or to increase its level of expression. In some cases, the desired mutation will be very specific (for example, the introduction of new restriction sites or changing a single amino acid at the active site of an enzyme). In other cases, it may be preferable to introduce random mutations into a small region of the vector (for example, when trying to increase the strength of a promoter). In either case, the most convenient way to mutagenize specific sites or regions of the vector is by PCR. Several of the most commonly used procedures will be described below.

#### 9.4.1 Introduction of specific mutations

All of the procedures to create non-random mutations begin with the synthesis of PCR primers which contain the desired mutation. Either a region of the plasmid or the entire plasmid is amplified, and the vector containing the mutation is reconstructed.

**Inverse PCR (IPCR) and enzymatic inverse PCR (EIPCR)**

An easy way to mutagenize a circular plasmid, inverse PCR procedures require only two primers to the complementary strands of the plasmid.
Their 5′ ends are adjacent to each other, and one of the primers contains the desired mutation (Helmsley et al., 1989). PCR with these primers will amplify the entire plasmid as a linear molecule, and this can then be recircularized with T4 DNA ligase and transformed into an E. coli host. Because the ends of the molecule are not always precisely blunt, it is often necessary to sequence the junction to confirm that no additional mutations have been introduced. A better way to accurately recircularize the plasmid is to use overlapping complementary primers containing a unique restriction site and digest the ends with the restriction enzyme before ligation (EIPCR). If a unique restriction site is located close enough to the mutation site, complementary primers containing both the restriction site and the mutation can be used for the PCR. Even if there are no restriction sites close to the mutation site, a unique class 2s restriction site whose recognition site is 5′ to the cutting site can be added as a 5′ extension to both primers.

Fig. 9.3. pWB20 Construction strategy.

1. Cut pFO20 with NcoI, fill in, and religate (= pFO20ΔNco). [Removes unwanted NcoI site from the PVA terminator region.]
2. Cut pUC19 with EcoRI + HinIII, add a linker, and ligate (= pUC19EB). Cut the plasmid with EcoRI and remove 5′-phosphates. [Removes all of the polylinker region, keeping only the EcoRI site and adding a BglII site.]
3. PCR the promoter region of pFO20ΔNco and cut the product with EcoRI + NcoI. [Adds an EcoRI and XbaI (with overlapping dam methylation) site to the 5′ end and an NcoI site at the translation start site.]
4. PCR the coding and terminator region of pFO20ΔNco and cut the product with EcoRI + NcoI. [Adds an EcoRI and XbaI site to the 3′ end, an NcoI site at the translation start site, and adds one amino acid to the N terminus of PVA.]
5. Ligate the three fragments from steps 2—4. [If the correct fragments come together, pWB20 is produced. The correct construct is identified by restriction analysis.]
Digestion of the amplified DNA with the class 2s restriction enzyme removes the recognition site and leaves complementary overhangs which are part of the original plasmid sequence. When ligated into a circle, the new plasmid is identical to the old one except for the introduced mutations.

**Recombination PCR (RPCR)**

Similar to IPCR, an entire plasmid is amplified with complementary primers which contain the desired mutation. Instead of recircularizing the plasmid in *vitro*, the linear DNA is transfected directly into competent *E. coli* cells (Jones and Howard, 1991). The host cells (including *recA* strains) contain recombinational repair enzymes which can recircularize the plasmid *in vivo*. Although initially simpler than IPCR or EIPCR, some errors can be introduced during this recombination, so further characterization of the plasmid is often advisable.

**Overlap PCR**

Introducing a mutation into the middle of a linear fragment of DNA requires four PCR primers. Two primers are complementary to the ends of the fragment, and two are complementary to each other and contain the desired mutation. Initially, two separate PCRs are carried out which amplify the two halves of the fragment. If the overlapping region containing the mutation also contains a unique restriction site, the two fragments can simply be digested with the enzyme and ligated together. If not, one alternative is to add a class 2s restriction site to the 5′ ends of the primers containing the mutation (Tomic *et al.*, 1990). When digested with the enzyme, the recognition sites are removed and the complementary overhangs contain only sequences from the original DNA fragment plus the mutation. A second alternative is to allow the overlapping region of the two fragments to prime each other in a third PCR reaction (Ho *et al.*, 1989). The two halves of the fragment are combined with the two primers which are complementary to the ends of the original DNA fragment and the PCR is repeated to regenerate the original fragment with the desired mutation. This approach can also be used to fuse together two unrelated gene fragments.

**9.4.2 Introduction of random mutations (mutagenic PCR)**

Depending upon the application, it may be preferable to introduce random mutations over a small region of an expression vector rather than create a larger number of plasmids with specific mutations. Mutagenic PCR takes advantage of the fact that native *Taq* DNA polymerase has an unusually high error rate (approximately $10^{-4}$ per nucleotide incorporated). This rate can be further increased by manipulating the concentration of MgCl$_2$, MnCl$_2$, and dNTP in the PCR reaction mix (Cadwell and Joyce, 1995). By carrying out a mutagenic PCR on a small region of an expression vector and subsequently
subcloning the fragment back into the vector, a large number of random mutations in a small region of the plasmid can easily be generated.

9.5 Characterization of High-producing Industrial Strains

Fungi have been employed in the fermentation industry for over 100 years. Traditional strain improvement methods such as random mutagenesis and selection have led to tremendous increases in the productivity of these organisms. The genetic changes that have resulted in these improvements, however, remain largely uncharacterized. PCR applications such as reverse transcription PCR (RT–PCR) and differential display RT–PCR (DDRT–PCR) enable us to identify genes which have altered expression levels in higher-producing organisms. Identifying these mutations enables the development of rational strategies for the genetic engineering of these strains for further improvements in productivity. Furthermore, knowledge gained regarding the genetic changes in one strain improvement programme may also help to speed productivity improvements of other related products. PCR can then be used for the identification and characterization of these genetically engineered fungi.

9.5.1 Characterization and quantitation of mRNA

RT–PCR is a technique for analysing gene expression by specific amplification of cDNA which has been synthesized from mRNA (reverse transcription). It can be used simply to detect the presence of a specific RNA transcript, or when a competitive template of a known quantity is added to the reaction, RT–PCR can be used to quantitate expression levels in RNA samples. The competitive template should be as similar to the cDNA target as possible. A cloned genomic fragment provides a good competitor if the PCR primers are chosen so that they span an intron. The amplified products can then be distinguished by the difference in size between the genomic and cDNA sequences. The advantage of RT–PCR over Northern blotting is that a smaller amount of total RNA of a lesser quality is required and further purification of poly(A)-tailed mRNA is not necessary. This is particularly helpful when dealing with fungal organisms from which RNA is difficult to extract.

In the case of the β-lactam antibiotics produced by *Penicillium chrysogenum* and *Acremonium chrysogenum*, most of the genes involved specifically in the biosynthetic pathway have been cloned (Martin *et al.*, 1994). In the higher producing strains, accumulation of beneficial mutations has increased the expression of these genes dramatically. Mutations in promoter regions can increase expression levels and alter the pattern of regulation that these genes display. Mutations in the coding region can affect the activity of the β-lactam biosynthetic enzymes, resulting in increased productivity of the
organism. One approach for analysing the mutations accumulated through the course of strain improvement uses PCR to amplify a defined region of genomic DNA from a number of different strains followed by a non-isotopic RNase cleavage assay to locate mutations (Myers et al., 1985). This technique locates the position of mutations so that sequencing can be limited to regions where mutations have been previously been identified. PCR primers designed to amplify a genomic region of 100–1000 bp in length are used to amplify a specific promoter or coding region from a wild-type or early development ‘reference strain’ and from higher-producing strains. These amplified products can be cloned into a vector between two different bacteriophage promoters. Separate sense and antisense transcripts can be generated by adding one of two different bacteriophage RNA polymerases. The sense strand amplified from the reference strain is annealed to the antisense transcripts generated from the high-producing strains. Mismatches in the complementary RNA templates will result in positions susceptible to RNase cleavage. By sizing the products on an agarose gel, the positions of these mismatches can be mapped, indicating the location of mutations incurred during strain improvement.

9.5.2 Differential display of fungal mRNA

The comparison of gene expression in fungal cells at various stages of differentiation has traditionally been performed by subtractive hybridization (Zimmerman et al., 1980). This technique is time consuming and requires the isolation of high quality mRNA. DDRT–PCR was developed to analyse a broad spectrum of expressed genes and to detect differences in expression levels between cell types (Appleyard et al., 1995). DDRT–PCR relies on the use of short random primers that non-specifically amplify fragments (<1000 bp) of cDNA. Differences in the expression of various genes can be determined by comparison of the DNA banding pattern observed upon amplification of different RNA samples. For example, antibiotics are produced by fungal organisms as secondary metabolites; specific conditions such as environmental stress are required for antibiotic production. DDRT–PCR of RNA isolated from cultures grown at either logarithmic phase (trophophase) or producing phase (idiophase) can be used to identify the genes that are expressed during the synthesis of antibiotics. These genes, related to antibiotic synthesis, are good targets for cloning and overexpression (Usher et al., 1992). For a company with a long history of strain improvement, analysis of high- and low-producing strains by this method can determine the genetic changes resulting in higher productivity. The DDRT–PCR and RT–PCR techniques are described in detail in other chapters of this book.

9.5.3 Characterization of transformants and integration sites

PCR is useful for identifying and characterizing fungal transformants. Because many selection systems for fungal transformation are not 100%
selective, abortive transformants due to the transitory uptake of transforming DNA can outgrow the selection. Fungal transformation in industrial applications is almost exclusively integrative. Therefore, to identify true transformants, genomic DNA is first isolated from putative transformants and a dot-blot or PCR amplification of the integrated DNA is performed. Nowak et al. (1995) used PCR to confirm that a linear DNA fragment which contains a mutant tubulin gene and lacks any bacterial sequences can confer resistance to benomyl and is sufficient for transformation of Acremonium chrysogenum.

The expression level of a heterologous gene which has been integrated into a fungal chromosome is often determined by the site of integration as well as by any regulatory sequences on the expression vector. A gene integrated into a transcriptional ‘hot spot’ can express at higher levels than the same gene inserted elsewhere on the chromosome. If a putative hot spot is identified after the random insertion of a vector, it may be desirable to isolate the flanking sequences from the host chromosome. These sequences could then be added to future expression vectors to direct integration to the hot spot by homologous recombination. Inverse PCR can be used to amplify the host DNA sequences flanking integrated transforming DNA. A Southern blot of the genomic DNA is first performed to identify restriction fragments which contain the integrated DNA and flanking genomic DNA. Sometimes a single fragment will contain both the upstream and downstream sequences, otherwise two separate fragments may be used for amplification of each flanking region. Once the restriction enzyme is chosen, genomic DNA is digested, diluted, and ligated so that the restriction fragments circularize. This material will serve as the template for the amplification. Unlike standard PCR where the primers are designed so that they amplify the sequences between them, primers for inverse PCR are designed to extend their template in outward directions. When annealed to the circular template, these primers will amplify a fragment that contains the sequences flanking the primers until they join at the restriction site used for cleavage of the genomic DNA. This amplified product can then be cloned and sequenced.

9.6 Future Applications

9.6.1 Efficient secretion of heterologous proteins

The efficiency of protein secretion is protein-specific and recombinant proteins are often difficult to secrete at high levels in fungal hosts. The effort at Genencor to produce bovine chymosin in A. nidulans and A. awamori demonstrated that transcription was not a limiting factor in chymosin production but that secretion may have been inefficient (Ward et al., 1990). The productivity and secretion of the chymosin protein was significantly improved by engineering a fusion protein product which links the cDNA
encoding bovine prochymosin in frame to the last codon of the *A. awamori* glycoamylase gene. The produced chymosin is autocatalytically released at pH 2 from the fusion protein after secretion (Ward *et al.*, 1990). This work demonstrated that secretion of the fusion protein is more efficient than for heterologous protein alone. If autocatalytic release does not occur in the fungal system, a cleavable linker peptide can be engineered between the proteins to allow separation of the products for purification. Site-specific mutagenesis by PCR or overlap PCR (Pont-Kingdon, 1994) can be used in the construction of fusion vectors and the generation of a cleavable linker.

### 9.6.2 Host improvement for heterologous gene expression

The ability of filamentous fungi to secrete large quantities of protein has made them an attractive host for the production of recombinant proteins. For the proper expression of therapeutic human proteins in filamentous fungi, several aspects need to be considered in addition to the efficient expression of heterologous genes:

- stability of the secreted proteins in the fermentation media;
- glycosylation pattern/ biological activity of the secreted proteins;
- ‘generally regarded as safe’ (GRAS) strain development.

**Proteinase-deficient mutants**

A recombinant protein secreted into the fermentation medium may be degraded by extracellular fungal proteinases. The aspartic proteinase of *A. awamori* had been shown to degrade secreted bovine chymosin and the gene encoding this enzyme was cloned (Berka *et al.*, 1990). Deletion of the native aspartic proteinase gene in *A. awamori* resulted in a significant reduction in overall extracellular proteinase activity. The production of chymosin was improved seven-fold by combining expression in the proteinase-deficient mutant with a classical strain improvement programme (Dunn-Coleman *et al.*, 1991). Based on the deduced amino acid sequence, *A. awamori* aspartic proteinase shares homology with other fungal aspartic proteinases (Berka *et al.*, 1990). Cloning of conserved protease genes in other fungal hosts by PCR could be performed as discussed earlier. Aspartic proteinase-deficient mutants could then be constructed by gene disruption or deletion. A number of genes encoding other fungal proteinases have been isolated (Van den Hombergh *et al.*, 1996), and some of these genes are highly conserved in fungi. Cloning of these genes can be facilitated by PCR and used to construct proteinase-deficient mutants.

**Glycosylation engineering**

The correct carbohydrate composition of many human therapeutic proteins is essential *in vivo* for their solubility, stability, and biological activity, as well as to avoid recognition by the human immune system. The recombinant mammalian proteins produced in yeast usually have a large number of
mannose residues (hyperglycosylation) which alter their serum half-life and antigenicity in mammals (MacKays, 1987). The glycosylated recombinant mammalian proteins produced in filamentous fungi usually co-migrate with the natural protein in SDS–PAGE gels, indicating no excess glycosylation. Upshall et al. (1987) have shown that human tissue plasminogen activator (tPA) produced in A. nidulans is correctly processed at the amino terminus and is not over-glycosylated. Ward et al. (1992) have also demonstrated that recombinant human lactoferrin expressed in A. oryzae has the same level of glycosylation as the authentic lactoferrin present in human milk. However, the nature and chemical composition of the carbohydrates added to these recombinant proteins derived from different fungal expression systems have never been characterized. When the cDNA encoding tPA was expressed by a strong fungal promoter in A. nidulans, the recombinant tPA protein was overglycosylated (Upshall et al., 1991). The authors suggested that in the high-yielding transformants, the translation of mRNA proceeds faster than the ability of the host cell to process and to translocate the protein extracellularly. The intracellular accumulation of this protein in the Golgi apparatus results in the continued addition of excess carbohydrate side-chains. A similar observation of over-glycosylation of recombinant proteins has been observed in the high-level expression of an aspartic proteinase derived from Rhizomucor miehei in A. oryzae, although the biological activity was not affected (Christensen et al., 1988). Therefore, in order to achieve the correct level of glycosylation, high-level expression of mammalian glycoproteins is not always desirable.

There is an increasing understanding of the biosynthetic pathways of protein glycosylation and the relationship between glycosylation and the biological activity of various proteins (Stanley, 1992; Jenkins and Curling, 1994). With this basic knowledge, a filamentous fungal host can be engineered by removing the fungal glycosyltransferases and adding the desired mammalian glycosyltransferases. Cloning, inactivation, and replacement of these genes can be achieved by applying PCR technology. Recently, Borsig et al. (1995) have demonstrated the expression of a functional human α-2,6(N)-sialyltransferase enzyme in S. cerevisiae. Theoretically, the same gene could be expressed in filamentous fungal cells.

**GRAS strain development**

For the production of recombinant proteins or metabolites by fungal organisms, it is important to use a fungal host which has a GRAS status. One of the most important factors that is required to recognize a fungal strain as safe is the absence of mycotoxins which are toxic to humans, animals, or plants. A number of fungal strains used for antibiotic and food industries are considered GRAS strains by the Food and Drug Administration (FDA) in the United States. These include A. awamori, A. oryzae, A. chrysogenum, P. chrysogenum and Fusarium graminearum A3/5. If a mycotoxin-producing fungus must be used to produce a desirable product, the cloning and
subsequent inactivation of genes encoding for one or more key enzymes in the mycotoxin biosynthetic pathway could be used to generate new GRAS strains. PCR will undoubtedly serve as a key technology for cloning and characterizing these genes.

9.7 Summary

This chapter details the many uses of PCR technology in the genetic engineering of industrial fungi for the commercial production of antibiotics, enzymes, secreted proteins and speciality chemicals. One of the major activities of industrial biotechnology process improvement laboratories is to overexpress the desired antibiotic, enzyme, or therapeutic protein using modern genetic engineering strategies through the construction of efficient expression vectors. PCR technology appears to be particularly useful in the development of improved vectors since most of the essential expression components are derived from existing vectors but require changes in flanking restriction sites. One of the highlights of this chapter is a detailed description of how PCR technology was used to overproduce penicillin V amidase (PVA) in engineered strains of *F. oxysporum*. When this host was transformed with a vector containing a tandem repeat representing two copies of the PVA gene, a nearly ten-fold improvement in enzyme production was noted.

PCR has also been shown to be a convenient procedure to mutagenize specific regions of an expression vector. Examples are described for the introduction of specific mutations using IPCR and EIPCR. The utility of RRPC, overlap PCR, and the use of PCR for the introduction of random mutations have also been highlighted in this chapter.

Another important application of PCR in fungal biotechnology is its use in characterizing high-producing industrial strains. Traditional strain improvement methodologies, including random mutagenesis and directed selection, have resulted in significant over-production of commercial metabolites. However, the genetic basis for these improvements remains largely uncharacterized. PCR strategies, including RT–PCR and differential DDRT–PCR, enable biotechnologists to identify discrete genes that may be tied to increased expression in higher-producing strains. These altered genes now provide for rational strategies for further improvements in strain productivity.

PCR applications will play even more important roles in future fungal biotechnology research and development programmes. Strategies have been described in this chapter on the efficient secretion of bovine chymosin through the fusion of the chymosin gene to the last codon of the *Aspergillus awamori* glucoamylase gene. Site-directed mutagenesis by PCR can be utilized for the generation of fusion vectors and cleavable linker protein products. Lastly, future uses of PCR and site-directed mutagenesis include the development of potential commercial, non-toxin-producing GRAS
fungal strains for the development of proteinase-deficient fungal isolates and for the development of fungal strains producing proteins with desirable changes in their protein glycosylation process.

References


10.1 Introduction

Lignin, cellulose and hemicellulose are the major structural polymers of plant biomass comprising, respectively, 45%, 20–30% and 20–30% of the dry weight of woody plants (Kirk and Farrell, 1987; Boominathan and Reddy, 1992). Because of the importance of lignocellulose as a renewable resource for the production of paper products, feeds, chemicals and fuels, there has been a considerable amount of research in recent years on the fungal degradation of lignocellulose (Kirk and Farrell, 1987; Boominathan and Reddy, 1992). Among the fungi, wood-rot basidiomycetes are considered the most efficient degraders of lignocellulose. White-rot fungi completely mineralize the three major lignocellulose polymers to CO₂, whereas the brown-rot fungi efficiently decompose cellulose and hemicellulose components of wood but mineralize lignin only to a limited extent (Kirk and Farrell, 1987; Reddy, 1993; Reddy and D'Souza, 1994). Earlier research focused extensively on lignocellulose degradation by the white-rot basidiomycete Phanerochaete chrysosporium (Kirk and Farrell, 1987; Boominathan and Reddy, 1992; Cullen and Kersten, 1992; Gold and Alic, 1993). However, there has been a growing interest recently in studying lignocellulose degrading systems of other wood-rot basidiomycetes (Higuchi, 1993; de Jong et al., 1994; Hatakka, 1994; Tuor et al., 1995).

An increasing number of researchers are currently utilizing molecular biological approaches to study fungal lignocellulose degradation (Cullen and Kersten, 1992; Reddy, 1993; Gold and Alic, 1993; Reddy and D’Souza, 1994). With the advent of the polymerase chain reaction (PCR) and its associated methodologies (Saiki et al., 1985; Mullis and Faloona, 1987; Innis et al., 1990;
Steffan and Atlas, 1991; Bej and Mahbubani, 1992; Dieffenbach and Dveksler, 1995), many researchers are using PCR-based strategies to better understand the physiology and molecular biology of fungal lignocellulose degradation and this is the focus of this review. A broad overview of the PCR techniques is presented first and is followed by a review of the applications of PCR methodology. The latter includes the detection, isolation and characterization of genes involved in lignocellulose degradation as well as studies on the expression of these genes under varying environmental conditions.

### 10.2 Extraction of Nucleic Acids for PCR Amplification

#### 10.2.1 From fungal mycelia

Most methods for DNA isolation from mycelia involve an initial lysis step using freeze–thaw cycles (Garber and Yoder, 1983), or by grinding the mycelia. Grinding is done with dry ice (Wahleithner et al., 1996), liquid nitrogen (Raeder and Broda, 1988; Lee and Taylor, 1990; Graham et al., 1994), liquid nitrogen and glass beads (Rao and Reddy, 1984), or glass beads only (van Vaerenbergh et al., 1995). This is followed by the use of phenol/chloroform/isoamyl alcohol extraction of the DNA and precipitation at a high salt concentration with isopropanol or ethanol (Sambrook et al., 1989). Gentle lysis of the mycelial cells by enzymatic hydrolysis (Black et al., 1989; Li and Ljungdahl, 1994) results in more intact DNA, but the yield is lower (van Vaerenbergh et al., 1995). Moreover, for use in PCR, mechanical shearing of the genomic DNA during isolation is not a major concern provided that the resulting DNA is ~10–20 kbp in size (Sogin, 1990; van Vaerenbergh et al., 1995). However, in cases where the same DNA preparations are used for both PCR and library construction, the DNA needs to be relatively pure. This is accomplished by extraction with phenol (Ashktorab and Cohen, 1992), cetyltrimethylammonium bromide (Kim et al., 1990; Graham et al., 1994), or by using affinity chromatography (e.g. Qiagen tip-500 mini-columns from Qiagen Inc., Los Angeles) (Wahleithner et al., 1996). In a recent study comparing various methods for extraction of DNA for PCR, it was reported that relatively pure DNA was needed even for PCR applications in order to remove inhibitors of Taq polymerase, especially from DNA samples of certain pigmented fungi (van Vaerenbergh et al., 1995). These researchers also found that the use of glass beads for DNA isolation reduced the amount of contaminating polysaccharides and inhibitors, and suggested that the freeze–thaw-based method for DNA isolation was superior to the other methods tested.

Fungal RNA isolation for use in PCR is based on standard protocols using a guanidinium isothiocyanate step (Lucas et al., 1977; Chirgwin et al., 1979; Timberlake and Barnard, 1981; Sims et al., 1988; Sambrook et al., 1989), followed by ethanol precipitation in the presence of high concentration of
salt (Sambrook et al., 1989; James et al., 1992), or isolation using affinity-based systems (Li and Ljungdahl, 1994; Wahleithner et al., 1996). Poly(A)-RNA is isolated from total RNA preparations using affinity column systems containing oligo-dT-cellulose (de Boer et al., 1987; Sambrook et al., 1989; Johnston and Aust, 1994a; Tempelaars et al., 1994), or magnetic beads (Brooks et al., 1993; Broda et al., 1995; Lamar et al., 1995).

### 10.2.2 From soil

Approaches used for the isolation of total DNA from soil, for PCR amplification of the fungal DNA component, are similar to those used for isolation and PCR amplification of soil bacterial DNA described previously (reviewed in Bej and Mahbubani, 1992; Picard et al., 1992; Tebbe and Vahjen, 1993; Moré et al., 1994; Selenska-Pobell, 1995; Van Elsas and Smalla, 1995). Johnston and Aust (1994b) used DNA extraction followed by PCR amplification for detecting fungal DNA in soils spiked with basidiomycetes. However, detection limits were low when compared to the use of template DNA isolated from pure cultures; this could be due to interference with the PCR amplification of the fungal DNA by the contaminating humic acids and DNA from other soil organisms (Tebbe and Vahjen, 1993; Tsai and Olson, 1993). In addition, clay in the soil can bind DNA and reduce the yield (Ogram et al., 1987). Another approach used is first to extract fungal cells from the soil and then extract the DNA; however, this approach is found to be less efficient (Hilger and Myrold, 1991). Recently, Thorn et al. (1996) described a procedure in which they used a selective medium for isolating diverse groups of soil basidiomycetes. However, it is not known what proportion of the total basidiomycetes in soil were isolated by this method. DNA isolated from selected pure cultures of soil basidiomycetes, utilizing the procedure of Thorn et al. (1996), is currently being used in PCR for the isolation of laccase and lip gene sequences (C.A. Reddy and T.M. D’Souza, unpublished work).

### 10.3 PCR Amplification Methods used in Studies on Lignocellulose Degradation

A number of papers have been published recently on the use of PCR methodology for a variety of studies on lignocellulose degradation by basidiomycetes. These studies include the detection, isolation and characterization of partial or complete lignocellulose degradative genes, the analysis of allele segregation, and gene expression under varying environmental conditions. PCR procedures used in these studies include DNA-PCR, RT-PCR, competitive RT-PCR, and inverse PCR. In this section, we provide a brief description of these procedures.
10.3.1 DNA-PCR

Replication of both strands of a target DNA sequence using a reaction mixture consisting of a thermostable DNA polymerase, forward and reverse oligonucleotide primers, the four deoxynucleotide triphosphates (dNTPs), magnesium ions and an appropriate template DNA is the standard procedure used for DNA-PCR (Saiki et al., 1985). The outcome is an exponential amplification of the specific target fragment, depending on the number of cycles of amplification performed (Saiki et al., 1985; Mullis and Faloona, 1987; Saiki et al., 1988; Vahey et al., 1995). Recent technologies have refined this basic PCR procedure to make it simpler and more efficient, resulting in enhanced amplification fidelity, higher specificity and minimizing DNA contamination problems.

In studies on lignocellulose degradation by fungi, conventional DNA-PCR procedures have been used to isolate and characterize lignin peroxidase (Johnston and Aust, 1994b; Collins and Dobson, 1995; Rajakumar et al., 1996; Schneider et al., 1996), manganese peroxidase (Asada et al., 1995), laccase (Giardina et al., 1995; Wahleithner et al., 1996; Yaver et al., 1996; D’Souza et al., 1996), cellobiohydrolase (Covert et al., 1992; Tempelaars et al., 1994), cellulase (Chow et al., 1994) and xylanase (Li and Ljungdahl, 1994) genes, as well as to analyse the allelic segregation patterns of some of these genes (Gaskell et al., 1992, 1994, 1995). The procedure has also been used to investigate the expression of lignocellulose degradative genes under varying environmental conditions (Covert et al., 1992; Brooks et al., 1993; Reiser et al., 1993; Broda et al., 1995; Gaskell et al., 1995; Rajakumar et al., 1996) as described in later sections in this chapter.

10.3.2 RT–PCR

RNA can also serve as a template for PCR amplification after conversion to cDNA (Todd et al., 1987; Veres et al., 1987). RNA-PCR, or RT–PCR (reverse transcriptase–PCR) as it is more popularly known, is a modified PCR procedure designed for analysing RNA transcripts. RT–PCR is more sensitive than other methods used for RNA analysis, such as Northern hybridization, S-1 nuclease analysis, RNase A protection assay and in situ hybridization (Kawasaki, 1990). The first step in this procedure is the synthesis of cDNA from RNA using reverse transcriptase. First strand cDNA is synthesized using either total RNA or mRNA as template. Random hexamers, the downstream primer, or oligo-dT can be used as primers, although the use of random hexamers consistently gives better amplification than the other two primers (Kawasaki, 1990). Since single RNA molecules can be amplified efficiently, even relatively crude RNA preparations can be used as the starting template for RT–PCR. The newly synthesized first strand cDNA is then used as a template for PCR, and the target fragment is amplified. In cases where the downstream primer is used for first-strand cDNA synthesis, it is necessary to add only the upstream...
primer to the reaction mixture. However, both primers should be used for PCR in cases where random hexamers or oligo-dT primers were used in the first-strand cDNA synthesis (Ausubel *et al.*, 1995). Contaminating DNA serving as the template is a common problem encountered in RT–PCR. Solutions to this problem include the use of primers positioned in separate exons (Kawasaki, 1990), or RNase-free DNase I digestion to remove the contaminating DNA (Ausubel *et al.*, 1995).

In studies on lignocellulose degradation by basidiomycetes, the RT–PCR technique has been used in the isolation of lignin peroxidase (Rajakumar *et al.*, 1996), manganese peroxidase (Matsubara *et al.*, 1996), laccase (Giardina *et al.*, 1995) and cellulbiohydrolase (Covert *et al.*, 1992) genes. The RT–PCR procedure has also been used in allele segregation studies (Gaskell *et al.*, 1995), and to investigate the expression of lignocellulose genes under different environmental conditions (Covert *et al.*, 1992; Brooks *et al.*, 1993; Johnston and Aust, 1994a; Broda *et al.*, 1995; Gaskell *et al.*, 1995; Lamar *et al.*, 1995; Bogan *et al.*, 1996b).

### 10.3.3 Competitive RT–PCR

Competitive RT–PCR is a technique that is commonly used for quantification of specific mRNA species (Gilliland *et al.*, 1990). This procedure involves co-amplification of a competing template that uses the same primers as those for the target cDNA, but the amplified products can be distinguished from each other as described below. Competitive templates can be either cDNA with a new restriction site, or genomic DNA containing an intron in the region to be amplified; the amplified product of the competitive template can then be differentiated from the amplified product of the target DNA based on size.

PCR amplification with genomic DNA as the competitive template may not be as efficient as that using cDNA because of the size increase and changes in duplex melting temperature (Gilliland *et al.*, 1990). Target cDNA should be co-amplified with serial dilutions of competitor DNA of known concentration. Since a change in any of the variables (such as polymerase, dNTPs, Mg²⁺, template DNA and primers) will affect the yield of target cDNA and the competitive template equally, relative ratios of the two should be preserved during amplification. The target cDNA concentration can then be estimated by direct scanning of ethidium bromide-stained gels or by measuring the radioactivity from incorporated radiolabelled dNTPs. Since the starting concentration of the competitive template is known, the initial concentration of the target cDNA can be determined fairly accurately. For example, it has been claimed that less than 1 pg of target cDNA from 1 ng of total mRNA can be accurately quantified using this procedure (Gilliland *et al.*, 1990).

The competitive RT–PCR procedure has been employed in studying the expression of lignin peroxidase (Stewart *et al.*, 1992; Lamar *et al.*, 1995), manganese peroxidase (Bogan *et al.*, 1996a) and cellulbiohydrolase (Lamar *et al.*, 1995) genes.
10.3.4 Inverse PCR

DNA sequences that are outside of the target DNA sequence flanked by the PCR primers can also be amplified by a modification of the basic PCR procedure designated ‘inverse PCR’. In this approach, the template is digested with a restriction enzyme that cuts outside the region to be amplified; the restricted template is then circularized by ligation, and the region outside the target sequence is amplified using primer sequences in the opposite orientation to those used for the initial PCR (Ochman et al., 1988; Triglia et al., 1988; Silver and Keerikatte, 1989). The inverse PCR method has been extremely useful in studying upstream and downstream regions of a target DNA segment, without the need to use conventional cloning techniques. It can also be used to prepare hybridization probes to identify and orient adjacent/overlapping clones isolated from a DNA library (Ochman et al., 1990). The inverse PCR technique has been used in isolating the cellobiohydrolase gene (Tempelaars et al., 1994).

10.4 Applications of PCR in Studies on Lignocellulose Degradation by Basidiomycetes

10.4.1 Detection, isolation, and characterization of genes involved in lignocellulose degradation

Wood-rot basidiomycetes are efficient degraders of the lignocellulose complex. The key enzymes involved are lignin peroxidases (LIP), manganese-dependent peroxidases (MNP), laccases, cellulases and xylanases. Several LIP (H1, H2, H6, H7, H8, and H10), and MNP (H3, H4, H5, and H9) isozymes produced by *Phanerochaete chrysosporium*, the prototype organism for studies on lignin degradation, have been described (Kirk and Farrell, 1987; Boominathan and Reddy, 1992; Cullen and Kersten, 1992; Gold and Alic, 1993). Recent studies have used PCR techniques to obtain a better understanding of the genes involved in lignocellulose degradation by *P. chrysosporium*, as well as a number of other wood-rot basidiomycetes.

**Lignin peroxidase genes**

Johnston and Aust (1994a) designed oligonucleotide primers H8I (forward) and H8II (reverse), corresponding to positions starting at 252 and 868 in the LIP H8 gene (Smith et al., 1988) of *P. chrysosporium* BKM-F 1767, for the detection of LIP H8 mRNA by RT-PCR (Table 10.1). Sequencing of a 402 bp PCR product verified the presence of H8 mRNA under N-limited or N-sufficient culture conditions. In another study, Johnston and Aust (1994b) used the same primers to detect *P. chrysosporium* in soil by PCR amplification using total DNA isolated from soil as the template. This resulted in the amplification of a 616 bp PCR fragment. However, they report that the method for detecting *P. chrysosporium* BKM-F 1767 using the H8 gene
primers was not as sensitive as that using the internal transcribed spacer (ITS) region of fungal ribosomal DNA (see Table 10.6).

Collins and Dobson (1995) described a method for PCR amplification and cloning of lip gene sequences from four different white-rot fungi, including *P. chrysosporium* ATCC 32629, *Chrysosporium lignosum* PM1, *Coriolus versicolor* 290, and *Bjerkandera adusta* DSM 3375. Genomic DNA was used as the template, with oligonucleotide primers corresponding to the conserved amino acid motifs present in the distal and proximal histidine regions of several *P. chrysosporium* LIP isozymes (Table 10.1). PCR-amplified products, ranging between 600 and 700 bp, were sequenced and found to have a nucleotide similarity of 70–80%. One of the PCR products was also found to have a 90% similarity with *GLG5*, which encodes the LIP isozyme H10 (Gaskell *et al.*, 1991).

PCR-amplification procedures were also used to determine the possible presence of lip gene sequences in genomic DNA of two white-rot basidiomycetes *Ceriporiopsis subvermispora* and *Phanerochaete sordida*, which were not known to produce LIPs (Rajakumar *et al.*, 1996). Degenerate oligonucleotide primers were prepared corresponding to amino acid motifs surrounding an essential histidine residue highly conserved in LIPs but not in MNPs (Table 10.1). Three genomic clones, two from *C. subvermispora*, and one from *P. sordida*, were obtained after cloning of the PCR-amplified products. These clones showed 92.6–95.6% amino acid similarity to lip A, which encodes the LIP isozyme H8 in *P. chrysosporium* (Gaskell *et al.*, 1994).

Schneider *et al.* (1996) used oligonucleotide primers specific for the H8-encoding *LPOA* gene of *P. chrysosporium* BKM-F 1767 (Holzbaur and Tien, 1988) to PCR-amplify a 1590 bp fragment, designated HG3, from the genomic DNA of this organism. Cloning and sequencing of HG3 showed that it is related to, but distinct from, *LPOA* (Holzbaur and Tien, 1988) and *GLG3* (Naidu and Reddy, 1990), both of which are known to encode LIP H8 in *P. chrysosporium*, and that HG3 is possibly a non-allelic variant of the *P. chrysosporium* BKM-F 1767 LIP H8 gene.

**Manganese-dependent peroxidase genes**

Asada *et al.* (1995) amplified mnp gene-specific sequences from genomic DNA and cDNA of *Pleurotus ostreatus* using PCR. Synthetic 30- to 32-mer primers (sequences not shown) that match the exon sequences around the proximal and distal His residues of the MNP of *P. chrysosporium* were used to obtain PCR products pcr1 using genomic DNA as the template, and pcr2 using cDNA as the template. Both pcr1 and pcr2 were cloned and sequenced. A subsequent PCR, using cDNA as the template, and a 32-mer upstream primer (sequence not shown) corresponding to the N-terminal amino acid sequence of the purified MNP, and a 30-mer downstream primer (sequence not shown) corresponding to a region near the 3′-end of pcr2, yielded a third PCR product, designated pcr3. Comparison of the deduced amino acid
### Table 10.1. Primers used for PCR amplification of lignin peroxidase-encoding genomic DNA and cDNA sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Organism</th>
<th>PCR procedure</th>
<th>5’ Primer</th>
<th>3’ Primer</th>
<th>Amplified product (bp)</th>
<th>Reference</th>
</tr>
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<td>LPOA</td>
<td>Phanerochaete chrysosporium</td>
<td>DNA-PCR</td>
<td>TGAGGCGCACAGTGCCCTGCT</td>
<td>GCAAGCTCGAGCTCCTGAACCT</td>
<td>616, 402</td>
<td>Johnston and Aust (1994a, b)</td>
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<td></td>
<td>(LIP H8) BKM-F 1767</td>
<td>RT–PCR</td>
<td>(H8I)</td>
<td>(H8II)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lip</td>
<td><em>P. chrysosporium</em> ATCC</td>
<td>DNA-PCR</td>
<td>T</td>
<td>T</td>
<td>500–700</td>
<td>Collins and Dobson (1995)</td>
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<td></td>
<td>32699, <em>Chrysosporium</em></td>
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<td></td>
<td><em>lignorum PM1</em>, <em>Coriolus</em></td>
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<td>adusta DSM 3375</td>
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<tr>
<td>lip</td>
<td><em>P. sordida</em> H8-8922,</td>
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<td>C A A</td>
<td>T</td>
<td>208–312</td>
<td>Rajakumar et al. (1996)</td>
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<td></td>
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<tr>
<td></td>
<td>(LIP H8)</td>
<td></td>
<td>G C A C G T C T C T C G C T C</td>
<td></td>
<td></td>
<td>Stewart et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>(LIP H8)</td>
<td></td>
<td>T C C A G T T C T C G T C</td>
<td>T C C A G T T C T C G T C G A G</td>
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<td>G C T G T C T T A C C C G G C G</td>
<td>T C C A G T T C T C G T C</td>
<td>677, 999</td>
<td>Stewart et al. (1992); Lamar et al. (1995)</td>
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<td></td>
<td>C T G G G C A T G G C A T T A T G A C C C</td>
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<td>T C C A G T T C T C G T C</td>
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<td>(LIP H10)</td>
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<td>T C C A G T T C T C G T C</td>
<td>T C C A G T T C T C G T C G A G</td>
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<td>lipF</td>
<td><em>P. chrysosporium</em> BKM-F 1767</td>
<td>RT–PCR</td>
<td>G C G G T A T C T C T T C C G C T</td>
<td>T C C A G T T C T C G T C</td>
<td>683, 1020</td>
<td>Stewart et al. (1992)</td>
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<td>P. chrysosporium</td>
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<td>GCCGCAATTCGCCCCCGCATG-</td>
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<td>Reiser et al. (1995)</td>
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<td>P. chrysosporium</td>
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<td>CAGCGCAATTCGCCCCCGCATG-</td>
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<td>Broda et al. (1992)</td>
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<td>Stewart et al. (1992)</td>
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<th>Gene</th>
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<th>3’ Primer</th>
<th>Amplified product (bp)</th>
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<td>lipD</td>
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<td>RT–PCR</td>
<td>TCCATCGCTATCTCGCCC</td>
<td>AFGCGAGCGAGAACCTGA</td>
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<td>(LIP H2)</td>
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<td>TGCCCTTGAGTCTCAAGG</td>
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<td>301, not given</td>
<td>Bogan et al. (1996b)</td>
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<td>301, not given</td>
<td>Bogan et al. (1996b)</td>
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<td>ACACCGCTGATGATTTGA</td>
<td>301, not given</td>
<td>Bogan et al. (1996b)</td>
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</tbody>
</table>

aLignin peroxidase isozymes encoded by the given *lip* genes (Kirk and Farrell, 1987; Boominathan and Reddy, 1992; Cullen and Kersten, 1992; Gold and Alic, 1993) are given in parentheses.
bSuperscripts in the ‘PCR procedure’ and ‘Amplified product’ columns refer to the corresponding superscripts in the ‘Reference’ column.
cThe upstream and downstream sequences of the primers for *LIG5* were erroneously switched in Table 1 of Broda et al. (1995). These sequences are shown correctly in this table and in Brooks et al. (1993).
sequence of PCR3 to the N-terminal amino acid sequences of MNPs revealed that it was part of an MNP gene. Using the cassette–primer PCR technique and a high fidelity Takara EX Taq polymerase, full-length sequences of genomic DNA and cDNA of the mnp gene were then obtained, cloned, sequenced and analysed. Their results showed that the P. ostreatus mnp gene is more closely related to the P. chrysosporium lip genes, with respect to sequence similarity and intron/exon organization, than to the mnp genes of this organism.

Full-length mnp genes were also obtained using PCR with primers (Table 10.2) corresponding to the region of the translational start and stop codons of mnp genes of P. chrysosporium BKM-F 1767 (Bogan et al., 1996a). These PCR-amplified MNP gene products served as templates in competitive RT–PCR experiments to analyse mnp transcripts of P. chrysosporium BKM-F 1767 during bioremediation of soils contaminated with poly-aromatic hydrocarbons (PAH) (described in section 10.4.3).

Matsubara et al. (1996) performed RT–PCR of poly(A)-RNA from the lignin-degrading deuteromycete IZU-154. DNA primers were synthesized corresponding to amino acid sequences around distal and proximal histidine residues in mnp genes of P. chrysosporium (Table 10.2). The resulting PCR fragments, approximately 400 bp in size, were isolated and labelled with digoxigenin using the DIG DNA labelling kit (Boehringer Mannheim), and were used to screen the cDNA library of IZU-154. Eight positive clones were obtained and separated into two groups based on restriction enzyme mapping and partial nucleotide sequences. Two cDNAs (IZ-MnP1 and IZ-MnP2), representative of the two groups, were further analysed. The coding sequences of IZ-MnP1 and IZ-MnP2 were 1152 bp (384 amino acids) and 1155 bp (385 amino acids), respectively, and showed 96.2% nucleotide and 95.1% amino acid similarities to each other. Also, IZ-MnP1 showed 72.5% nucleotide and 79% amino acid similarity to P. chrysosporium mnp1 cDNA (Pribnow et al., 1989).

**Laccase genes**

Laccase, in addition to its role in lignin biodegradation (Bourbonnais and Paice, 1990; Hatakka, 1994), also has important applications in the bleaching of wood pulp (Bourbonnais and Paice, 1992) and the degradation of phenolic compounds (Bollag et al., 1988; Roy-Arcand and Archibald, 1991). The structure and function of fungal laccases were reviewed by Thurston (1994). A number of researchers have used PCR as a tool in their studies on the laccase genes of wood-rotting basidiomycetes (Giardina et al., 1995, 1996; Yaver et al., 1996; Wahleithner et al., 1996; D’Souza et al., 1996) and these studies are reviewed in this section.

Giardina et al. (1995) used RT–PCR and the rapid amplification of cDNA ends (RACE) technique (Frohman et al., 1988) to amplify partial cDNA fragments encoding the laccase pox1 gene from the white-rot basidiomycete Pleurotus ostreatus. After first-strand cDNA synthesis using
Table 10.2. Primers used for PCR amplification of manganese peroxidase-encoding genomic DNA and cDNA sequences.

<table>
<thead>
<tr>
<th>Gene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Organism</th>
<th>PCR procedure</th>
<th>5’ Primer</th>
<th>3’ Primer</th>
<th>Amplified product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mnp-1</td>
<td>Pleurotus ostreatus IFO 30160</td>
<td>DNA-PCR</td>
<td>Not given</td>
<td>Not given</td>
<td>Not given</td>
<td>Asada et al. (1995)</td>
</tr>
<tr>
<td>mnp-1</td>
<td>P. chrysosporium</td>
<td>DNA-PCR</td>
<td>GCAATGGGCCCTCGGTTCCTC</td>
<td>TTAGGCAGGCGGCACTGA</td>
<td>Not given</td>
<td>Bogan et al. (1996a)</td>
</tr>
<tr>
<td>mnp-2</td>
<td>P. chrysosporium</td>
<td>DNA-PCR</td>
<td>CAGATGGCCTTCAAGTCCTC</td>
<td>TTATCGGGAACGTTGGAACGT</td>
<td>Not given</td>
<td>Bogan et al. (1996a)</td>
</tr>
<tr>
<td>mnp-3</td>
<td>P. chrysosporium</td>
<td>DNA-PCR</td>
<td>GCCATCAAGCCAGCGCAATG</td>
<td>TGTCGGGCGCTAGACTTA</td>
<td>Not given</td>
<td>Bogan et al. (1996a)</td>
</tr>
<tr>
<td>MNP</td>
<td>Fungus IZU-154,</td>
<td>RT–PCR</td>
<td>ATCCGCCCTACCTTCCACCA</td>
<td>GCGACGAGTGGGAGCCGAG</td>
<td>~400</td>
<td>Matsubara et al. (1996)</td>
</tr>
<tr>
<td>mnp-1 (=mnp-2)</td>
<td>P. chrysosporium</td>
<td>RT–PCR</td>
<td>TCGGCTAAGGCCCTTGATATCCAG</td>
<td>GCCAGTCGTTGCGGCGGCGAG</td>
<td>517, 676</td>
<td>Broda et al. (1996)</td>
</tr>
<tr>
<td>mnp-1</td>
<td>P. chrysosporium</td>
<td>RT–PCR</td>
<td>CAGACGGGTACCGGCGGTCACC</td>
<td>AGTGCGGAGGGGCGACATCAC</td>
<td>Not given</td>
<td>Bogan et al. (1996a)</td>
</tr>
<tr>
<td>mnp-2</td>
<td>P. chrysosporium</td>
<td>RT–PCR</td>
<td>CCGACGCGCACCAGCGTCAGC</td>
<td>CGACGCGGAGGGGCGGACGGC</td>
<td>Not given</td>
<td>Bogan et al. (1996a)</td>
</tr>
<tr>
<td>mnp-3</td>
<td>P. chrysosporium</td>
<td>RT–PCR</td>
<td>CCGACGCTAAGGGCTACAC</td>
<td>AGCGGCGACGGCGGCGGACC</td>
<td>Not given</td>
<td>Bogan et al. (1996a)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Manganese peroxidase isozymes encoded by the given mnp genes (Kirk and Farrell, 1987; Boominathan and Reddy, 1992; Cullen and Kersten, 1992; Gold and Alic, 1993) are shown in parentheses.
reverse transcriptase (RT) and the RT primer, another primer was used to amplify a 750 bp cDNA fragment corresponding to the 3′-end of the *pox1* gene (Table 10.3). A second PCR was carried out using two new primers and the first-strand cDNA as template to amplify a 900 bp cDNA fragment corresponding to the 5′-end of the *pox1* gene. Except the RT primer, the primers were derived from the genomic DNA sequence determined earlier for the *pox1* gene (Table 10.3). Together the two PCR fragments obtained accounted for the entire *pox1* cDNA. The deduced amino acid sequence of *pox1* showed 45–63% similarity with the laccase genes of *Coriolus hirsutus*, basidiomycete PM1 (CECT 2971), *Phlebia radiata* and *Agaricus bisporus*, but only 20–27% similarity with the laccase genes of *Neurospora crassa*, *Aspergillus nidulans* and *Crpyhnonectria parasitica* (Giardina et al., 1995). In order to determine whether *pox1* and *pox2* (another *P. ostreatus* laccase gene) were allelic or not, two separate PCR amplification experiments were performed using the genomic DNA from six monokaryotic isolates as the templates. The two primer sets were located at identical positions on both *pox1* and *pox2* sequences, thus resulting in the amplification of 570 bp fragments (Table 10.3). However, the two products were distinguishable because of the presence of a unique *Hin*dIII site in *pox1*, and a unique *Bam*HI in *pox2*. The results showed that the two genes segregated together into monokaryons and hence were non-alleles (Giardina et al., 1995).

In a subsequent study, Giardina et al. (1996) used RT–PCR to synthesize the entire laccase *pox2* cDNA sequence, as well as the genomic equivalent of the *pox2* gene. RT–PCR was done using mRNA and the oligonucleotide primer dT-NotI (Table 10.3). This was followed by PCR with the high fidelity *Pfu* polymerase, using the primer pairs O1/O2, LAC1/LAC2, and LAC3/dTNotI (Table 10.3), at annealing temperatures of 50°C, 60°C and 53°C, respectively. To amplify the 3′-end of the *pox2* gene, primer pairs LAC3/LAC4 (Table 10.3) were used with *Pfu* polymerase, genomic DNA as template, and an annealing temperature of 60°C. The *pox2* cDNA had 84% nucleotide and 90% amino acid similarity with the *pox1* cDNA (Giardina et al., 1996).

Yaver et al. (1996) used RT–PCR and two degenerate oligonucleotide primers (Table 10.3), one based on the N-terminal sequence of laccase and the other based on the C-terminal sequence of the *C. hirsutus* gene, to amplify a laccase gene-specific PCR product from *Trametes villosa* using first-strand cDNA synthesized from poly(A)-RNA of xylidine-induced cells. The first-strand cDNA was synthesized using reverse transcriptase and a commercial cDNA synthesis kit (Gibco BRL). The PCR product was then used as a probe to screen a cDNA library of *T. villosa* and obtain more than 100 positive clones. Out of these, the longest clone (LCC cDNA) was sequenced and found to have an identity of 90% with the sequence of the *C. hirsutus* laccase.

A 220 bp PCR-amplified fragment, isolated from genomic DNA of the phytopathogenic fungus *Rhizoctonia solani* with oligonucleotide degenerate primers, was used to isolate laccase gene sequences from a *R. solani* genomic
Table 10.3. Primers used for PCR amplification of laccase-encoding genomic DNA and cDNA sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Organism</th>
<th>PCR procedure</th>
<th>5' Primer</th>
<th>3' Primer</th>
<th>Amplified product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laccase</td>
<td>Pleurotus ostreatus (3'-pox)</td>
<td>RT–PCR</td>
<td>CGCAGATCCCAACTTGGGATCGACTGGCTT</td>
<td>AATTCGCGGCGCGCTTTTTTTTTTTTTTTTTT</td>
<td>750</td>
<td>Giardina et al. (1995)</td>
</tr>
<tr>
<td>Laccase</td>
<td>Pleurotus ostreatus (5'-pox1)</td>
<td>RT–PCR</td>
<td>ATGTTTCCAGGCGGACGG</td>
<td>CCAGTCGATCCCAAGTTGGG</td>
<td>900</td>
<td>Giardina et al. (1995)</td>
</tr>
<tr>
<td>Laccase</td>
<td>Pleurotus ostreatus (5'-pox2)</td>
<td>DNA-PCR</td>
<td>GCTGGCACGTTGTAAG</td>
<td>TCTGCTTAACGCCCTGC</td>
<td>570</td>
<td>Giardina et al. (1995)</td>
</tr>
<tr>
<td>Laccase</td>
<td>Pleurotus ostreatus (3'-pox1)</td>
<td>RT–PCR</td>
<td>CGCTGCACTGTCATGAGACAGATG [LAC1]</td>
<td>TCTGCTTAACGCCCTGC</td>
<td>842</td>
<td>Giardina et al. (1996)</td>
</tr>
<tr>
<td>Laccase</td>
<td>Species</td>
<td>Method</td>
<td>Primer</td>
<td>Sequence</td>
<td>Accession</td>
<td>References</td>
</tr>
<tr>
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</tr>
<tr>
<td>Laccase</td>
<td><em>Trametes villosa</em></td>
<td>RT–PCR</td>
<td>ACCAGNCTAGACCGGGNTGAGAPCT-</td>
<td>CGCGGCCGCT AGGA T CCT CACAA TGGCCAA-</td>
<td>Not given</td>
<td>Yaver et al. (1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GAGCNGAAGCCGACTTGCTGTCACT-</td>
<td>GTCTTGCCCTCGACCTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ACTGACGNGAAGCGGACTTGCTGGT</td>
<td>CACTATCTTGCAAGATGTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DNA-PCR</td>
<td>T</td>
<td>ATCCAFTGGCATGQNTTTTTCAA</td>
<td>GTONCAATGATACCAGAANGT</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ACCAGAANGT A GGTG G A</td>
<td>GTGGCTATGATACCAGAANGT</td>
<td>144–217</td>
</tr>
<tr>
<td>Laccase</td>
<td><em>Rhizoctonia solani</em></td>
<td>DNA-PCR</td>
<td>TCC</td>
<td>CATTGGCATGQNTTTTTCAC</td>
<td>CATTGGCATGQNTTTTTCAC</td>
<td>A C C C G G A</td>
</tr>
<tr>
<td>Laccase</td>
<td><em>Fomes fomentarius</em></td>
<td>DNA-PCR</td>
<td></td>
<td>CATTGGCATGQNTTTTTCAC</td>
<td>CATTGGCATGQNTTTTTCAC</td>
<td>A C C C G G A</td>
</tr>
</tbody>
</table>

*Superscripts in the ‘Gene’ column refer to the corresponding superscripts in the ‘Reference’ column.*
DNA library (Wahleithner et al., 1996). The primers corresponded to two amino acid sequences conserved among laccases (IHWHGFFQ and TFWYHSH; Table 10.3). The sequence of the cloned PCR fragment had 60% similarity to the corresponding region of a C. hirsutus laccase gene (Kojima et al., 1990). Sequencing of 15 other PCR clones established the presence of three distinct laccase genes, designated lec1, lec2, and lec3. Using the cloned PCR fragment as the probe, nine genomic clones were isolated from the R. solani genomic library.

D’Souza et al. (1996) used primers (Table 10.3), based on the conserved sequences around the two pairs of histidines in the N-terminal copper-binding regions of known basidiomycete laccases, to rapidly isolate laccase gene-specific sequences from different wood-rot basidiomycetes. Genomic DNA was isolated from each of the wood-rot fungi and used as the template in PCR. Of the 11 white-rot and brown-rot fungi (belonging to nine genera) used for the amplification of the laccase gene-specific PCR products, eight gave amplification products of approximately 200 bp. One strain, Gloeophyllum trabeum Mad-617-R, a brown-rot fungus, gave a 144 bp PCR product. No PCR products were obtained for Fomes fomentarius and Pleurotus ostreatus, two white-rot fungi which are known to produce laccases (Hatakka, 1994). However, Southern hybridization of restriction enzyme-digested genomic DNA of F. fomentarius and P. ostreatus, using the G. trabeum 144 bp PCR product as the probe, showed hybridization bands indicating that laccase genes are present in these two strains. Several of the white-rot fungi tested gave two PCR products, one of 144 bp, and the other of the expected 200 bp. Sequencing of the larger PCR product revealed a size range of 197–217 bp; the smaller PCR products all were 144 bp in size (D’Souza et al., 1996). All the PCR products sequenced had a high degree of similarity to corresponding regions of previously published laccase gene sequences. Alignment of the predicted amino acid sequences of the PCR-amplified products showed high similarities between the laccase gene segments of the wood-rotting fungi and lower similarities to those of Aspergillus nidulans and Neurospora crassa, two laccase producing non-wood rotters (Fig. 10.1).

Following up on the demonstration of a laccase gene-specific PCR product from the genomic DNA of the brown-rot fungus G. trabeum, D’Souza et al. (1996) demonstrated for the first time the presence of laccase activity in cultures of this and other brown-rot fungi. Using the approach described above, a laccase gene-specific 199 bp PCR product was recently isolated and sequenced from a soil basidiomycete, AX-1 (Srinivasan et al., 1996). The laccase gene fragment of AX-1 showed a high degree of nucleotide similarity (62–94%) to the corresponding portions of laccase genes from wood-rotting basidiomycetes.

**Cellulase genes**

A large number of microorganisms degrade cellulose in nature; of these, fungi are perhaps the most studied. Cellulolytic enzymes include the
hydrolytic cellulases and oxidative cellulases. Hydrolytic cellulases consist of three classes: endoglucanases (EG), cellobiohydrolases (CBH) and \( \beta \)-glucosidases. Oxidative cellulases consist of two classes: cellobiose quinone oxidoreductase (cellobiose dehydrogenase) and cellobiose oxidase (Eriksson et al., 1990; Béguin and Aubert, 1995). Fungal and bacterial cellulases share similar functional characteristics, including a catalytic core, a conserved cellulose-binding terminal region, and an intervening, highly glycosylated hinge region (Knowles et al., 1987; Gilkes et al., 1991; Bayer et al., 1996). A brief review of the use of PCR methodology for studying the cellulases of ligninolytic fungi is presented here.

Covert et al. (1992) characterized three structurally related CBH genes \( cbh1-1, cbh1-2 \) and \( cbh1-3 \) from \( P. \) chrysosporium which were closely related to the \( T. \) reesei \( cbh-1 \) gene (Shoemaker et al., 1983). Full-length cDNA species encoding \( P. \) chrysosporium \( cbh1-1, cbh1-2 \) and \( cbh1-3 \) were amplified by PCR, cloned, and sequenced. RT–PCR was carried out using total RNA isolated from \( P. \) chrysosporium BKM-F 1767 cellulolytic

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**Fig. 10.1.** Alignment of the predicted amino acid sequences of the PCR-amplified laccase gene products (D’Souza et al., 1996). The abbreviations for the fungal strains are: \( Tv \), Trametes versicolor; \( Pb \), Phlebia brevispora; \( Gl103561 \) and \( Gl58537 \), Ganoderma lucidum strains 103561 and 58537; \( Le1 \) and \( Le2 \), Lentinula edodes PCR products 1 and 2; \( Lt \), Lentinus tigrinus; \( Gt \), Gloeophyllum trabeum; \( Tvi \), Trametes villosa; \( Po \), Pleurotus ostreatus; \( PM1 \), unidentified basidiomycete; \( Ch \), Coriolus hirsutus; \( Cv \), Coriolus versicolor; \( Pr \), Phlebia radiata; \( Ab \), Agaricus bisporus; \( An \), Aspergillus nidulans; \( Ne \), Neurospora crassa. Sequences from the latter nine organisms were taken from published literature (see D’Souza et al., 1996). Alignment was done using the clustalw program (Riverside Scientific Enterprises). The program introduces gaps where necessary to optimize the alignments. Amino acid positions with a ≥ 50% match are highlighted. Invariant amino acid positions are shown in bold.
cultures using downstream primers (Table 10.4) to synthesize first-strand cDNA of cbh1-1 and cbh1-3. Then, upstream primers were used to amplify the cDNA for these two genes. The cDNA cbh1-2 was amplified by DNA-PCR employing double-stranded cDNA (synthesized using an oligo-dT primer) as the template and two specific primers (Table 10.4) (Covert et al., 1992). A highly conserved region, assumed to be the catalytic site, within the P. chrysosporium cDNAs cbh1-1, cbh1-2 and cbh1-3 was 80%, 69% and 80% similar, respectively, to the corresponding region within the T. reesei cbh-1 gene. However, analysis of the P. chrysosporium cbh1-1 gene revealed that it was different from other fungal cellulase genes because it did not contain the hinge or tail regions. Transcription studies on these three genes were also carried out using competitive RT–PCR (described in Section 10.4.3).

Two allelic variants of the P. chrysosporium ME-446 cbhII gene were isolated from a cDNA library after screening with a T. reesei cbhII gene probe (Tempelaars et al., 1994). Characterization of these cDNAs by restriction enzyme analysis, Southern hybridization analysis, and partial sequencing using the deoxy chain-termination method, revealed that they were very similar, and in the cellulose-binding domain had a 65% amino acid similarity to the corresponding region of the T. reesei cbhII gene. The results also revealed that the entire cbhII gene is present on a PstI genomic fragment. Therefore, inverse PCR (Ochman et al., 1990) was used to amplify a 1.1 kbp fragment from PstI-digested and religated genomic DNA, using two primers (Table 10.4) deduced from the cbhII cDNA sequence, and the high fidelity Pfu polymerase. The inverse PCR was followed by conventional DNA-PCR using the 1.1 kbp PCR-amplified DNA fragment as the template and two primers, based on the DNA sequence adjacent to the PstI restriction site. The resulting 2.3 kbp PCR-amplified genomic cbhII fragment was cloned, sequenced, and analysed. Sequence comparison showed that both the genomic and cDNA clones represent two distinct classes (type 1 and type 2), which were later shown to be allelic variants (Tempelaars et al., 1994).

Chow et al. (1994) isolated two allelic forms of a cellulase (endoglucanase) CEL3-encoding cDNA from a cDNA library of A. bisporus, an important commercial mushroom. The cDNA library was constructed in lambda ZAP-PII (Raguz et al., 1992) from cDNA synthesized from a cellulose-grown mycelial culture. A sample of this cDNA library was excised in vivo as pBluescript plasmids with R408 helper phage (Short et al., 1988), and total plasmid DNA was isolated from the bacterial culture harbouring these plasmids. PCR amplification was performed on the total plasmid DNA using a 20-mer degenerate oligonucleotide (OL60B1, Table 10.4), which is complementary to a region from a 19 kDa CNBr CEL3-cleaved peptide, as the reverse primer, and the T7 primer (Table 10.4) which corresponds to a region 5' of the insertion site in the pBluescript plasmid, as the forward primer. Three PCR amplified products, 400 bp, 600 bp and 1.1 kbp in size, were obtained. Northern hybridization studies showed that the 400 bp PCR
### Table 10.4. Primers used for PCR amplification of cellulase-encoding genomic DNA and cDNA sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Organism</th>
<th>PCR procedure</th>
<th>5' Primer</th>
<th>3' Primer</th>
<th>Amplified product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cbh1-1</td>
<td><em>P. chrysosporium</em></td>
<td>RT–PCR</td>
<td>GAGAATCTGCAAACCGCTACCAT</td>
<td>TGAATCCACCAAATATCGCGAGG</td>
<td>Not given</td>
<td>Covert et al. (1992)</td>
</tr>
<tr>
<td>cbh1-2</td>
<td><em>P. chrysosporium</em></td>
<td>DNA–PCR</td>
<td>GAGAAATCGAGTGAGACCGGATA</td>
<td>TTTGTGACCCAGCATAGACAA</td>
<td>Not given</td>
<td>Covert et al. (1992)</td>
</tr>
<tr>
<td>cbh1-3</td>
<td><em>P. chrysosporium</em></td>
<td>RT–PCR</td>
<td>GAGAATCTACCCGTCTGCTCACT</td>
<td>GAGAATCTCCATTAGCTGCTGGGA</td>
<td>Not given</td>
<td>Covert et al. (1992)</td>
</tr>
<tr>
<td>cbh1-1</td>
<td><em>P. chrysosporium</em></td>
<td>Competitive RT–PCR</td>
<td>CACAGTTGCTCAGGAGGATGTC [pr6]</td>
<td>CAAAAGCCCGTGTTGGAGGT</td>
<td>221, 274</td>
<td>Covert et al. (1992)</td>
</tr>
<tr>
<td>cbh1-2</td>
<td><em>P. chrysosporium</em></td>
<td>Competitive RT–PCR</td>
<td>GCCCGAGTGGACTGCT</td>
<td>CGTGGTAGCCAGCACCTT [pr5]</td>
<td>132, 197</td>
<td>Covert et al. (1992)</td>
</tr>
<tr>
<td>cbh1-3</td>
<td><em>P. chrysosporium</em></td>
<td>Competitive RT–PCR</td>
<td>GCTAAGTACGCGGCTGGCGCTA</td>
<td>GCAGTCTGGTTCGGAGAGCAG</td>
<td>226, 286</td>
<td>Covert et al. (1992)</td>
</tr>
<tr>
<td>cbhl</td>
<td><em>P. chrysosporium</em></td>
<td>Inverse PCR</td>
<td>GACGTCTAGGCTGCTAGATAACGG</td>
<td>ACAAAGCTTGGCGCTCCGTGACCC</td>
<td>1100</td>
<td>Tempelaars et al. (1994)</td>
</tr>
<tr>
<td>cbhl</td>
<td><em>P. chrysosporium</em></td>
<td>DNA–PCR</td>
<td>GCGGAATTCAGCGATCCACATCATCACTGCTGCTG</td>
<td>CGTGGAGTGGAGAAGCCTGAAATTCGGCATT-CAACCGG</td>
<td>2300</td>
<td>Tempelaars et al. (1994)</td>
</tr>
<tr>
<td>Gene</td>
<td>Organism</td>
<td>PCR procedure</td>
<td>5’ Primer</td>
<td>3’ Primer</td>
<td>Amplified product (bp)</td>
<td>Reference</td>
</tr>
<tr>
<td>------</td>
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</tr>
<tr>
<td>cbh1.2</td>
<td><em>P. chrysosporium</em> ME446 (ATCC 34541)</td>
<td>DNA-PCR</td>
<td>CACTCTTCGATCTACCTTGCT</td>
<td>CTGCCGGTCTCGGTATCATGTC</td>
<td>Not given, 560, 840</td>
<td>Tempelaars et al. (1994), Broda et al. (1995)</td>
</tr>
<tr>
<td>cbh1</td>
<td><em>P. chrysosporium</em> ME446 (ATCC 34541)</td>
<td>DNA-PCR</td>
<td>CTCAAGCTCTTACACGC</td>
<td>CCAAACCTACACTACACGC</td>
<td>Not given, 950, 1330</td>
<td>Tempelaars et al. (1994), Broda et al. (1995)</td>
</tr>
<tr>
<td>cel3</td>
<td><em>Agaricus bisporus</em> D649</td>
<td>DNA-PCR</td>
<td>TGAATTCACTACGACAGAAAATG</td>
<td>GTAACGACTACTACTAGGCG</td>
<td>400, 500, 1100</td>
<td>Chow et al. (1994)</td>
</tr>
<tr>
<td>cbh1-4</td>
<td><em>P. chrysosporium</em> BKM-F 1767 (ATCC 24725)</td>
<td>RT–PCR</td>
<td>GATGCAATTGTTTCGGGGCCGGCCGGCGAT</td>
<td>GACTGAAATTCTAGTACACTGCGAATGT</td>
<td>1537</td>
<td>van Rensburg et al. (1996)</td>
</tr>
<tr>
<td>cbh1-1</td>
<td><em>P. chrysosporium</em> BKM-F 1767</td>
<td>RT–PCR</td>
<td>TTCCTTTTGGGTCGCCCTGC</td>
<td>ACCTCCAAACGCGGCTG</td>
<td>766, 8176</td>
<td>Lamar et al. (1995)</td>
</tr>
<tr>
<td>cbh1-4</td>
<td><em>P. chrysosporium</em> BKM-F 1767</td>
<td>RT–PCR</td>
<td>AAGGTGGTTCTCGACTGAA</td>
<td>CTCCAGGCTCTACCGTGC</td>
<td>741, 7795</td>
<td>Lamar et al. (1995)</td>
</tr>
</tbody>
</table>

*aSuperscripts in the ‘Amplified product’ column refer to the corresponding superscripts in the ‘Reference’ column.*
product hybridized to a 1.4 kbp cel mRNA; the 600 bp product to a 1.4 kbp mRNA as well as to a small mRNA thought to be non-cel mRNA. The 1.1 kbp PCR-amplified product was assumed to be an almost full-length copy of the cel3 cDNA. The 400 bp PCR product was digested with EcoRI to remove a small terminal vector sequence, labelled with 32P-dCTP, and used as a probe to screen the cDNA library (Chow et al., 1994) to obtain 14 cDNA clones which were then analysed. The nucleotide sequence of the clones showed that two types of cDNAs were isolated. Using genomic DNA from four monokaryotic mycelia, the segregation pattern of the cel3 gene was studied. Since two differing patterns of hybridization were obtained in Southern hybridization analysis, it was concluded that the two genes were allelic (Chow et al., 1994). These alleles had 98.8% sequence identity to each other.

A cDNA fragment encoding the P. chrysosporium cellobiohydrolase (cbh1-4) was amplified using RT–PCR and cloned (van Rensburg et al., 1996). First-strand cDNA was synthesized from P. chrysosporium BKM-F 1767 poly(A)-RNA using a commercial kit and commercial primers (Boehringer Mannheim). The DNA was then amplified using two primers (Table 10.4), CBH-5' (forward primer), and CBH-3' (reverse primer) based on a previously published cbh1-4 gene sequence (Vanden Wymelenberg et al., 1993). The 1537 bp PCR-amplified product was determined to be identical to the published nucleotide sequence of cbh1-4, minus the two introns (Vanden Wymelenberg et al., 1993). It was subcloned into the yeast multicopy episomal plasmid pJC1 by inserting it between the yeast phosphoglycerate kinase-I gene promoter (PGK1P) and terminator (PGK1T) sequences. The recombinant plasmid (pCBH) contained the PGK1P-cbh1-4-PGK1T construct, designated CBH1. A 3477 bp PvuII DNA fragment containing this CBH1 construct was excised from pCBH and subcloned into a unique SmaI site of another plasmid, pEND. This plasmid consisted of the yeast multicopy episomal, expression/secretion vector (pDLG4) and the gene end1, which encodes the endo-β-1,4-glucanase of Butyrivibrio fibrisolvens, a cellulolytic rumen bacterium. The resulting 12 kbp plasmid, designated pENCB, was transformed into the yeast Saccharomyces cerevisiae, and the coexpression of cbh1-4 and end1 was analysed. The maximum cellulolytic activity of the transformed S. cerevisiae Y294 [pENCB], containing both the genes, was 1380 U as compared to S. cerevisiae with cbh1-4 (which had a maximum cellobiohydrolase activity of 12.03 U) and end1 (which had a maximum endo-β-1,4-glucanase activity of 1100 U). These studies indicated that coexpression of both the genes results in enhanced cellulose degradation (van Rensburg et al., 1996).

Cellobiose dehydrogenase (CDH) is a haemoflavoenzyme, secreted under cellulolytic conditions, that oxidizes cellohexaoses, lactose and mannotriose (Eriksson et al., 1990). The role of CDH in wood degradation, and catabolism of cellobiose has been suggested (Bao et al., 1993; Eriksson et al., 1993; Ander, 1994). In a recent study, Raices et al. (1995) isolated more
than 50 putative positive clones, using either immuno- or gene-probe screening for the CDH gene. Out of these clones, five were sequenced and found to overlap with identical sequences, but lacking the 5′-end of the mRNA. This 5′-end was obtained by RACE (Frohman et al., 1988), using poly(A)-RNA as the template and two nested primers (sequences not shown) based on the 5′-end of the longest cDNA isolated. A single PCR product, Ra-12, corresponding to the missing 5′-end, was obtained. By determining the sequences of the 5′ (Ra-12) and 3′ (CP3) sections of the CDH gene, the complete cDNA sequence was determined (Raices et al., 1995). The full-length cDNA was assembled by combining Ra-12 and CP3 in a recombination PCR using the Ra-12 forward and the CP3 reverse primers (sequences not shown). The complete cDNA contains 2310 translated bases excluding the 3′ poly(A)-tail. Analysis of the CDH amino acid sequence showed the presence of haem and flavin adenine dinucleotide (FAD) domains, as well as the nucleotide-binding motif. The FAD domain appears to be distantly related to the glucose/methanol/choline (GMC) oxidoreductase family (Raices et al., 1995).

Xylanase genes
Graham et al. (1993) used a novel PCR-based procedure to synthesize an artificial xylanase gene from the white-rot fungus Schizophyllum commune. The sequence of the synthetic gene was designed so that restriction enzyme recognition sequences could be introduced into the gene without alteration of the amino acid sequence of the final translation product. Additionally, the design also incorporated a selection of codon bias suited for expression in E. coli. Overlapping PCR primers Xyl1 (189-mer) and Xyl2 (208-mer) were mixed with different dilutions of two other primers, Xyl3 (42-mer) and Xyl4 (36-mer), in a PCR-amplification procedure to yield a 426 bp double-stranded product XylA, representing two-thirds of the 5′-end of the xylanase gene. An aliquot of XylA was mixed with a 217-mer oligonucleotide primer and flanking primers Xyl3 (5′-end) and Xyl6″ (3′-end) and subjected to a second PCR to yield a 629 bp double-stranded XylB DNA representing the full-length xylanase-encoding xynA gene. An aliquot of XylA was mixed with a 217-mer oligonucleotide primer and flanking primers Xyl3 (5′-end) and Xyl6″ (3′-end) and subjected to a second PCR to yield a 629 bp double-stranded XylB DNA representing the full-length xylanase-encoding xynA gene. The XylB fragment was subcloned into a plasmid vector; after sequencing three subclones, it was found that errors were introduced in the sequences of each of the subclones because of the low fidelity of Taq polymerase. The error was corrected by selecting one of the subclones and excising the segment containing the error using flanking restriction sites and replacing it with a correct segment from another subclone; the result was an authentic xynA gene. The synthesized xynA gene was subcloned into the NheI–HindIII site of the protein expression vector, pTUG. To accomplish this, the 5′ PstI site of xynA was changed to a NheI site by PCR amplification using a new 5′ primer Xyl3′ (with a NheI recognition site) and the original 3′ primer Xyl6″. The PCR product was then subcloned into pTUG and, after verification that the sequence was correct, was transformed into E. coli JM101. Xylanase-
secreting clones produced clearing zones when grown on Luria broth agar plates containing remazol brilliant blue xylan substrate. Using the data obtained from the N-terminal sequence of purified xylanase APX-II from the xylanolytic fungus *Aureobasidium pullulans* Y-2311-1, Li and Ljungdahl (1996) designed two degenerate biotinylated oligonucleotide primers, P0813 and P3035 (Table 10.5) to amplify a xylanase gene from genomic DNA. The resulting 83 bp biotinylated PCR fragments were used as probes to screen a cDNA library, as well as in the characterization of genomic DNA (Southern blot), and in the study of regulation of the xylanase gene (Northern blot). Five positive clones were obtained after screening the cDNA library of which two were sequenced. The sequence data revealed that none of these clones contained the 5′-end of the gene. Using PCR, the 5′-end of the cDNA was amplified from the cDNA library, using two oligonucleotide primers. The forward primer (T3 promoter sequence; P200) was located at the 5′ of the insert while the reverse primer (P3338) was 152 bp from the 5′-end of the positive cDNA clones (Table 10.5). The PCR product was cloned and sequenced and using the sequence data, two oligonucleotide primers PFW and PRW (Table 10.5) were synthesized. PFW and PRW corresponded to the 5′- and 3′-ends of the full-length cDNA for the *A. pullulans* xylanase, and were used to amplify the whole gene. Comparison between the genomic and cDNA sequences revealed that one intron of 59 bp was present in the coding region. The result of the Southern blot showing the presence of only a single copy in the genome, and that of the Northern blot showing that only a single transcript was present, indicated that the two xylanases present in the culture were encoded by the same gene (Li and Ljungdahl, 1996).

10.4.2 Allele-specific oligonucleotides

The PCR approach was also used to differentiate *lip* alleles of *P. chrysosporium* (Gaskell et al., 1992, 1994, 1995). Two primers, A and B (Table 10.1), were used to amplify *lip* H8 sequences from the genomic DNA of basidiospores (homokaryotic) and dikaryote isolates of *P. chrysosporium*. Amplified products of 251 bp and 260 bp were obtained. These PCR products were Southern blotted and probed with gene-specific oligonucleotide probes to identify rapidly the H8 gene and its alleles (Gaskell et al., 1992).

In another study, Gaskell et al. (1994) developed a strategy for genetic mapping by segregation analysis. They monitored allelic segregation by PCR of genomic DNA from single basidiospore-derived cultures followed by probing with allele-specific oligonucleotides. Gene-specific PCR primers were used for 12 *P. chrysosporium* genes coding for *LIP* (Table 10.1), glyoxal oxidase (GLOX) and CBH (Table 10.4). Based on the sequences of the amplified products, allele-differentiating probes were prepared. Genetic linkage was determined by monitoring the segregation of specific alleles.
Table 10.5. Primers used for PCR amplification of xylanese-encoding genomic and cDNA sequences.

PCR
procedure

Gene

Organism

Xylanese
[xylA =
5′-end of
xynA]

Schizophyllum DNA-PCR
commune

5′ Primer

3′ Primer

ACCCCGTCTTCTACCGGTACCGACGGTGGTTACTACTACAGCTGGTGGACCGACGGTGCTGGTGACGCTACCTACCAGAACAACGGTGGTGGTTCTTACACGTTAACCTGGTCTGGTAACAACGGTAACTTAGTTGGTGGTAAAGGTTGGAACCCGGGTGCTGCTTCTAGATCTATCTCTTACTCTGGT
[Xyl1] (189-mer)

GACGGAGCGTTGTAACGCCAGGTAGACAGGATGTCGTAGGTGGCGCCGTTGCAGGTAACGGATCCTTTGTGAGAAGCAGCAGAAGACGGGTCGTAAGAACCGTAAGATTCAACGATGTAGTATTCGATCAGACTCGAGCGGGTCCAACCGTAAACAGACAGGTAAGAGTTACCGTTCGGCTGGTAGGTACCAGAGTAAGAGATAGATC
[Xyl2] (208-mer)

ACCGCCTCTGCAGCTTCTGGTACCCCGTCTTCTACCGGTAC
[Xyl3] (42-mer)
Xylanase
[xylB =
xynA]

Schizophyllum DNA-PCR
commune

ACCGCCTCTGCAGCTTCTGGTACCCCGTCTTCTACCGGTAC
[Xyl3] (42-mer)

CTGGGTACCATCGATAGACGGAGCGTTGTAACGCCA
[Xyl4] (36-mer)
CTAGACGGTGATGGTAGCGGTACCAGAAGACTGGTAACCTTCGGTCGCGACGATCTGGTAGTTGTGTTCAGAACCCAGGTTCATACCCAGGCCTTTCCAAGCGTCGAAGTGGCACTGAACGTCGACGGTACCAGAGATAGAACCACCCGGAGCTTTTTTCGGGTTACGTACAGACCAGAACTGTTCGAAGGTCTGGGTACCATCGATAGACGGAGCGTTGTA
[Xyl5] (222-mer)
CGTATAAGCTTCTATTAGCTAGCGGTGACGGTGATGGTAGCCG
[Xyl6″] (43-mer)

Amplified
product
(bp)
Reference
426

Graham et al.
(1993)

629

Graham et al.
(1993)


<table>
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<tr>
<th>Xylanese</th>
<th>Schizophyllum DNA-PCR commune</th>
<th>Xylanase</th>
<th>Aureobasidium DNA-PCR pullulans Y-2311-1</th>
<th>Xylanase</th>
<th>Aureobasidium DNA-PCR pullulans Y-2311-1</th>
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<tr>
<td>xynA</td>
<td></td>
<td>3′-end of</td>
<td></td>
<td>5′-end of</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>xynA</td>
<td></td>
<td>xynA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Y-2311-1</td>
<td></td>
<td>Y-2311-1</td>
<td></td>
</tr>
<tr>
<td>[xynA]</td>
<td></td>
<td>[3′-end of</td>
<td></td>
<td>[5′-end of</td>
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<tr>
<td></td>
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<td>xynA</td>
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<td>pullulans</td>
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<td></td>
<td></td>
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<td>Y-2311-1</td>
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<td></td>
<td></td>
<td>[Y XL3]</td>
<td></td>
<td>[P0813]</td>
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<tr>
<td></td>
<td></td>
<td>(35-mer)</td>
<td></td>
<td>[P200]</td>
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<td>[Xyl3]</td>
<td></td>
<td>[PFW]</td>
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<td>(43-mer)</td>
<td></td>
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<tr>
<td>CGCTCTGCTAGGGTAGACCCGGTCTTACCCGGTAC</td>
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<td>GTAGCAAGGTTCGACAT</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>C G TATA AGCTTCTA TT AGCT AGCGGTGACGG</td>
<td></td>
<td>TGA TGTTAGCCG</td>
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<tr>
<td></td>
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<td>[Xyl6] (43-mer)</td>
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<tr>
<td>Given</td>
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<td></td>
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<tr>
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<td></td>
<td></td>
<td>[Xyl6] (43-mer)</td>
<td></td>
<td>[Xyl6] (35-mer)</td>
</tr>
</tbody>
</table>

Li and Ljungdahl (1994)
among homokaryotic segregants. Homokaryotic single-basidiospore cultures were first isolated and identified, followed by the extraction of genomic DNA, amplification of genes, and differentiation of alleles using radiolabelled oligonucleotides as probes. The linkage was computed from allelic co-segregation frequencies. Five linkage groups were identified, one of which contained eight closely linked \textit{lip} genes. One unlinked \textit{lip} gene, was determined to co-segregate with a \textit{cbh1} gene cluster (Gaskell \textit{et al.}, 1994).

Recently, Gaskell \textit{et al.} (1995) investigated the copy number, inheritance, genomic location, and distribution of Pce1, an insertional element within \textit{P. chrysosporium} gene \textit{lipI}, discovered after analysis of the sequences of the ten known \textit{lip} genes. PCR was used to amplify a 1767 bp Pce1 insertional element from genomic DNA of \textit{P. chrysosporium} BKM-F 1767, using primers at the junction of the insertion point of Pce1 in \textit{lipI2}. The Pce1 element was localized to the 3.7 Mb chromosomal band by experiments using the amplified product to probe Southern blots of clamped homogeneous field electrophoresis gels of size-fractionated chromosomes of a dikaryotic strain of \textit{P. chrysosporium} BKM-F 1767. The copy number and distribution of Pce1 were assessed by Southern blot analysis of restriction enzyme-digested genomic DNA. An additional Pce1-like sequence was found on a 4.6 kbp \textit{Bam}HI–XbaI genomic DNA fragment. Southern blot analysis of genomic DNA from 17 strains of \textit{P. chrysosporium} using Pce1 as a probe showed that only three isolates hybridized under moderate stringencies. One widely used laboratory strain, ME-446, did not show hybridization with Pce1. Allelic segregation patterns were determined for \textit{lipA} and \textit{lipI} using PCR with gene-specific primers and 69 single-basidiospore isolates. PCR products of 2236 bp and 479 bp allowed easy differentiation of \textit{lipI} alleles. For \textit{lipA}, allele-specific probes were used to differentiate PCR products, since all the \textit{lipA} gene-specific PCR products were identical in length. The results showed that \textit{lipA} and \textit{lipI} alleles co-segregate. RT–PCR followed by probing with allele-specific oligonucleotides, was also done in order to detect \textit{lipI1} and \textit{lipI2} transcripts in N-limited and C-limited cultures (discussed in section 10.4.3).

10.4.3 Gene expression under varying environmental conditions

Stewart \textit{et al.} (1992) used competitive RT–PCR and gene-specific oligonucleotide probes to study the pattern of LIP gene expression in \textit{P. chrysosporium} BKM-F 1767. First-strand cDNA of four closely related LIP genes (\textit{lipA}, \textit{lipB}, \textit{GLG5} and 0282) was prepared by reverse transcription using a conserved downstream primer (Table 10.1). Gene-specific upstream primers were used to amplify the double-stranded cDNA. Each PCR contained genomic DNA as a competitive template. The cDNA levels of the four genes were quantified in N-limiting and C-limiting culture conditions. The transcript levels for the LIP H8-encoding \textit{lipA} and \textit{lipB} genes, and for the
LIP H2-encoding 0282 gene, were similar in C- and N-limited cultures. However, for the LIP H10-encoding GLG5 the N-limited cDNA levels were between 25 and 100 pg, but no cDNA product was amplified in the C-limited cultures. Competitive PCR was also used to quantify the LIP H2-encoding GLG4 and V4 transcript levels, using gene-specific primers (Table 10.1). The GLG4 transcript was more abundant (1000-fold) in C-limited than in N-limited cultures. The V4 gene transcript could only be detected using a second set of primers (Table 10.1) and when reverse transcription reactions included 0.5 µg of RNA and were extended to 45 min. Under these conditions, the V4 gene transcripts were found to be more abundant in N-limited than in C-limited cultures.

Brooks et al. (1993) determined the expression profiles of LIP H8-encoding LIG1 and LIP H2-encoding LIG5 in P. chrysosporium ME-446 cultures under conditions of nitrogen limitation (LN) or non-limitation (HN) using gene-specific primer pairs (Table 10.1). No LIG1 expression was seen in either LN or HN cultures, but LIG5 expression was seen in both LN and HN cultures. In carbon non-limiting cultures, however, no LIG5 transcripts were seen, leading to the conclusion that LIG5 expression in strain ME-446 is dependent on low C concentration.

Brooks et al. (1993) determined the expression profiles of LIP H8-encoding LIG1 and LIP H2-encoding LIG5 in P. chrysosporium ME-446 cultures under conditions of nitrogen limitation (LN) or non-limitation (HN) using gene-specific primer pairs (Table 10.1). No LIG1 expression was seen in either LN or HN cultures, but LIG5 expression was seen in both LN and HN cultures. In carbon non-limiting cultures, however, no LIG5 transcripts were seen, leading to the conclusion that LIG5 expression in strain ME-446 is dependent on low C concentration.

The expression of LIP genes from C-limited cultures of P. chrysosporium BKM-F 1767 was analysed by PCR. For this, cDNA prepared from 6-day-old C-limited cultures was used as the template with degenerate oligonucleotide primers corresponding to the regions surrounding the proximal and distal His residues in LIP from BKM-F 1767 (Reiser et al., 1993). Twenty-four PCR clones were analysed and found to be ordered into two sets, based on digestion with the restriction enzymes SalI and RsaI. One set showed a high degree of similarity to the LIP H2-encoding CLG4 cDNA sequence of de Boer et al. (1987), while the other was similar to the LIP H8-encoding L18 cDNA sequence (Ritch et al., 1991). These represented the major transcripts in C-limited cultures.

Broda et al. (1995) applied the RT–PCR technique to monitor the expression of 11 genes from low-N cultures of P. chrysosporium ME-446 during growth with one of four different carbon sources: low glucose (0.2%), high glucose (2.0%), Avicel (0.2%) and ball-milled straw (BMS; 0.2%). Different primers were used for amplification of LIP H8-encoding genes LIG1, LIG2, LIG3, and LIG4, the LIP H2-encoding gene LIG5, the unknown LIP-encoding gene LIG6 (Table 10.1), the MNP-encoding gene mnp1 (Table 10.2), and the CBH-encoding genes cbh1.1, cbh1.2, and cbhII (Table 10.4). Amplification of the constitutively expressed trpC gene was used as a positive control for gene expression in all cultures (primers for trpC are shown in Table 10.6). Cultures grown in low glucose expressed only LIG5 (on days 3–8), and cbh1.1 (on days 7 and 8). High glucose cultures expressed none of the genes studied, except low levels of mnp1 on day 3. Avicel cultures expressed cbh1.1 and cbh1.2 on days 3–6; LIG1 and LIG2 on day 6, LIG3 on days 4–8, LIG6 on day 3, and cbhII, LIG5 and mnp1 on days...
<table>
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<th>Gene</th>
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<th>PCR procedure</th>
<th>5’ Primer</th>
<th>3’ Primer</th>
<th>Amplified product (bp)</th>
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<td>495, 640</td>
<td>Lamar et al. (1995)</td>
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<td>126, 179</td>
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*Presented here as an example of similar papers published using the internal transcribed spacer (ITS) region or other regions of fungal ribosomal DNA for phylogenetic studies of lignocellulosic fungi.*
3–8. BMS cultures expressed *cbh1.1* and *cbh1.2* on days 3–6, *LIG1* on day 8 and *cbhII, LIG5* and *mnp1* on days 3–8.

RT–PCR and allele-specific oligonucleotide probes were used to detect the LIP H2-encoding *lipI* transcripts in N-limited and C-limited cultures of *P. chrysosporium* BKM-F 1767 (Gaskell *et al.*, 1995) using gene-specific primers (Table 10.1). The fungus expressed *lipII* transcripts in both culture media, but *lipI2* transcripts were not detected; the *lipI2* contains a 1747 bp insertional element Pce1 (described in Section 10.4.2). The results indicated that Pce1 inactivated the *lipI2* gene.

Rajakumar *et al.* (1996) used RT–PCR to determine whether LIP-like genes were transcribed in *P. sordida* and *C. subvermispora* cultures grown under ligninolytic (N-limited medium) conditions. The primers used are shown in Table 10.1. The results showed that one LIP-like sequence, with a 88.4% nucleotide similarity to the corresponding region of a *T. versicolor* lip gene, was transcribed in *P. sordida* cultures. However, no LIP-like transcripts were detected in *C. subvermispora* cultures.

Quantitative RT–PCR analysis was done on mRNA isolated from anthracene-transforming soil cultures of *P. chrysosporium* BKM-F 1767 to assess the temporal regulation of the *H2O2*-producing glyoxal oxidase (GLOX) gene expression (Bogan *et al.*, 1996b). Anthracene, a known LIP substrate, needs the presence of *H2O2* for its transformation by LIP. The primers used for PCR amplification of the *lip* gene sequences are shown in Table 10.1; no primer sequences for *glx* were given. The results showed that during the initial period of incubation (<10 days), the LIP H8-encoding *lipA* and the LIP H2-encoding *lipD* transcripts were at the highest levels. The highest transcript level for any of the genes studied was that of the LIP H2-encoding *lipI* (days 15–20). The *lipA* transcript level, however, maintained high levels for a majority of the 25 days. No *lipF* (unknown LIP-encoding) transcripts were seen on any of the days studied. The transcript levels of *lipA* and *glx* genes correlated with the levels of the enzymes they coded for (H8 and GLOX, respectively). The oxidation of anthracene was also seen to take place throughout the course of the study (Bogan *et al.*, 1996b).

In a separate study, Bogan *et al.* (1996a) used competitive RT–PCR to examine the temporal expression of three MNP genes (*mnp-1*, *mnp-2* and *mnp-3*) of *P. chrysosporium* BKM-F 1767 during the bioremediation of PAH-contaminated soil. All three transcripts were seen on day 1 or 2, with peak levels on day 6. The β-tubulin gene (*tub*) and glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) transcripts were also determined by competitive RT–PCR as controls for mRNA loading. The results showed that *mnp* mRNA levels correlated with MNP enzyme levels and with the disappearance of PAHs from the soil, supporting the hypothesis that MNP are involved in the oxidation of PAHs in soil (Bogan *et al.*, 1996a).

Competitive RT–PCR was used to determine the relative transcript levels of *P. chrysosporium* BKM-F 1767 *cbb1-1, cbb1-2* and *cbb1-3* genes in
glucose-grown, sucrose-grown and cellulose–cellobiose-grown cultures (Covert et al., 1992). First-strand cDNAs were prepared by reverse transcription with downstream primers (see Table 10.4). This was followed by PCR-amplification using upstream primers (Table 10.4) in the presence of serial dilutions of genomic DNA as competitive templates. The primers flanked an intron common to all three genes, so that the PCR-amplified products obtained from cDNA and genomic DNA could be identified on agarose gels. Gene-specific oligonucleotide probes were used to further establish the identity between the three genes. The results showed that cbh1-3 transcripts were about 1000-fold more abundant than those of cbh1-1 and cbh1-2 in cellulose–cellobiose-grown cultures. The cbh1-1 and cbh1-2 transcript levels were low in all four cultures, but were higher than cbh1-3 transcript levels in 3-day-old glucose-grown cultures. Thus, unlike cbh1-3, cbh1-1 and cbh1-2 appear not to be subjected to glucose repression.

10.5 Future Perspectives

An increasing number of researchers have been using PCR methodology in recent years for studies on fungal lignocellulose degradation. The techniques applied to date have been limited to DNA-PCR, RT-PCR, competitive RT–PCR and inverse PCR. These techniques have been used for the detection, isolation and characterization of genes involved in lignocellulose degradation. The latter included genes encoding LIP, MNP, laccase, cellulase and xylanase. PCR approaches have also played a major role in the study of regulation of expression of genes encoding lignocellulose-degrading enzymes under a variety of environmental conditions. Furthermore, PCR techniques were also useful for mapping and segregation analysis of genes involved in lignin and cellulose breakdown as well as for studying the molecular genetics of an insertional element Pce1 in P. chrysosporium.

The PCR approaches described above have been useful in enhancing our understanding of fungal lignocellulose degradation but these studies represent only the beginning of what we believe will be an expanding field in future research. Several new and improved PCR-based techniques show promise for future applications in this research area. For example, DDRT–PCR (Liang and Pardee, 1992; see Chapter 3) is a sensitive technique that could be used for detailed studies on environmental control of gene expression. Another area where PCR could be used effectively would be in phylogenetic studies of lignocellulose-degrading wood-rot basidiomycetes (Hibbett and Vilgalys, 1991; Kwan et al., 1992; Boysen et al., 1996; Bunyard et al., 1996; Chiu et al., 1996). More recently, in the Center for Microbial Ecology at Michigan State University, we have initiated studies using denaturing gradient gel electrophoresis PCR (DDGE–PCR) to characterize the phylogenetic affiliation of the various lignin-degrading soil basidiomycetes. Thus it appears quite likely that a greater variety of PCR approaches
will be used by researchers in the future for increasing the knowledge of fungal lignocellulose degradation systems.

Acknowledgements

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11.1 Introduction

Nearly every food or feed commodity can be contaminated by fungal organisms. It is estimated that approximately 25% of the yearly production of plant-derived foods are spoiled by fungi. Many of the food-borne filamentous fungi are capable of producing mycotoxins, which are toxic metabolites of concern to both the health of humans and the health of animals. Mycotoxins, like antibiotics, are secondary metabolites, which are primarily produced in the idiostage of fungal growth. Some mycotoxins, such as patulin, were originally isolated as antibiotics due to their inhibitory activity towards bacteria, but were later shown to be toxic in animal experiments (Wilson, 1974). Mycotoxins belong to different chemical classes, and mainly polyketide, isoprene and amino acid-containing substances are found (Martin, 1992). Approximately 300 different mycotoxins have been identified, but only about 20 mycotoxins produced by different species are relevant to human health (Table 11.1). Due to the different molecular structures of these mycotoxins, their influences on human and animal health range from teratogenic, immunosuppressive, tremorgenic, nephrotoxic, hepatotoxic to carcinogenic effects (Bullerman, 1979). Some mycotoxicoses are documented by epidemiological studies. Examples are the ergotism due to the ingestion of ergot alkaloids produced by *Claviceps purpurea* (Barger, 1931), the alimentary toxic aleukia which occurred in the Urals and was due to the infection of cereals with T2 toxin-producing fungi (Bamburg et al., 1968), the endemic nephropathia in the Balkan region which correlated with the high occurrence of ochratoxin A-producing filamentous fungi (Krogh, 1987), the high incidence of oesophageal cancer in relation to the high intake...
of fumonisin-containing maize products (Nelson et al., 1993), and some cases of epidemiological correlations between the higher incidence of liver cancer and higher ingestion of aflatoxins (Chu, 1991). Even if these drastic, well-documented cases are rare, the constant uptake of small amounts of mycotoxins, especially those with carcinogenic activity, can have profound effects on human health.

This situation demonstrates the importance of using appropriate methods to control the mycological status of food and feed commodities. Conventional methods for the detection of mycotoxinogenic fungi are culture methods, which are time-consuming and require expertise in fungal taxonomy. Taxonomic classification can be simplified by the use of selective media, but the time required is not reduced (Davies et al., 1987). These problems can be overcome by the use of the PCR which can reduce the detection time from several days to several hours. The diagnostic PCR approach for the detection of mycotoxinogenic fungi is an indirect method. DNA or DNA fragments of the target organism can be amplified and detected by gel electrophoresis. However, the method does not distinguish between dead and living cells. This fact, which is a disadvantage for the detection of pathogenic bacteria in foods, is advantageous in the case of

<table>
<thead>
<tr>
<th>Genus</th>
<th>Toxins</th>
</tr>
</thead>
</table>
| Aspergillus | Aflatoxin  
Sterigmatocystin  
Cyclopiazonic acid |
| Penicillium | Patulin  
Ochratoxin A  
Citrinin  
Penitrem A  
Cyclopiazonic acid  
PK toxin |
| Fusarium | T2 Toxin  
Deoxynivalenol  
Nivalenol  
Zearalenon  
Diacetoxyscirpenol  
Fumonisin |
| Alternaria | Tenuazonic acid  
Alternariol  
Alternariol monomethylether |
| Claviceps | Ergot alkaloids |

Table 11.1. Mycotoxins of relevance to human health.
mycotoxinogenic fungi, as mycotoxins are usually very stable. A positive PCR can, therefore, be taken as an indication that the sample potentially contains mycotoxins and should be analysed further. The PCR approach, however, has the disadvantage that the quantification of fungal biomass is not easy and although some PCR-based quantification methods have been described (Hu et al., 1993), they are too laborious for routine analysis.

A prerequisite for the development of a diagnostic PCR is the availability of unique target sequences, which are specific for the mycotoxin-producing fungus. Ideally, these target sequences should not be present in strains of the same species that are not able to produce mycotoxins. Possible target sequences for PCR or gene probe approaches are given in Table 11.2. For detection of pathogenic or toxinogenic bacteria in foods, respective toxin or virulence genes are often used as target sequences for amplification (Olsen et al., 1996). The counterparts for mycotoxinogenic fungi are the genes which code for the enzymes of the mycotoxin biosynthetic pathway (referred to as mycotoxin biosynthetic genes). However, to date, only some of the genes of mycotoxin biosynthetic pathways have been cloned and sequenced. The best analysed biosynthetic pathways at the genetic level are those from the aflatoxins (Trail et al., 1995a; Yu et al., 1995), the trichothecens (McCormick et al., 1996), patulin (Beck et al., 1990; Wang et al., 1991), PR-toxin (Procter and Hohn, 1993) and for sterigmatocystin (Kelkar et al., 1996). Sterigmatocystin is a precursor of aflatoxin and the sterigmatocystin biosynthetic genes are rather homologous to the aflatoxin biosynthetic genes (Brown et al., 1996). Genes for biosynthetic enzymes of secondary metabolites are usually clustered (Hohn et al., 1993) and this is true for the aflatoxin (Trail et al., 1995a; Yu et al., 1995), sterigmatocystin (Brown et al., 1996), trichothecene (Hohn et al., 1993) and fumonisin biosynthetic genes (Desjardins et al., 1996).

The polyketide synthase gene from *Penicillium patulum*, which is the key enzyme in the biosynthetic pathway of the mycotoxin patulin, has been cloned (Beck et al., 1990). Polyketide synthases are often involved in fungal secondary metabolism and can be found in various species (Hopwood and Sherman, 1990). The polyketide synthases are composed of different catalytic domains, each specific to a certain reaction. These domains, however, often

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Target sequences</th>
<th>Microorganisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene specific</td>
<td>Toxin genes, virulence genes</td>
<td>Bacteria</td>
</tr>
<tr>
<td></td>
<td>Toxin biosynthetic genes</td>
<td>Fungi</td>
</tr>
<tr>
<td>Empirical</td>
<td>Ribosomal genes, random cloned fragments, RAPD products</td>
<td>Bacteria and fungi</td>
</tr>
</tbody>
</table>
share high sequence homology, even between species, which makes this gene unsuitable as a specific target gene for diagnostic PCR (Mayorga and Timberlake, 1992).

11.2 Mycotoxin Biosynthetic Genes as Target Sequences

11.2.1 Aflatoxinogenic fungi

The aflatoxins are the most carcinogenic natural substances known and can be found in various food commodities (Diener et al., 1987). They are produced predominately by Aspergillus flavus, A. parasiticus and A. nomius (Bennett, 1988). Whereas nearly all A. parasiticus isolates are able to produce aflatoxins B₁, B₂, G₁ and G₂, A. flavus is only able to produce aflatoxins B₁ and B₂. Only about 40–50% of A. flavus strains isolated are able to produce these mycotoxins. A. flavus is highly related taxonomically to A. oryzae as is A. parasiticus to A. sojae (Kurtzman et al., 1986) and they share 90% nucleotide sequence homology. A. oryzae and A. sojae are used in the food industry as fermentation organisms and both are generally recognized as safe (GRAS) organisms. Both species have never been reported to produce aflatoxin, but they show high morphological similarity to their aflatoxin-producing relatives (Klich et al., 1995). A method specific to aflatoxin-producing fungi should be capable of differentiating between these closely related species and also between aflatoxin-producing and non-producing A. flavus strains.

The aflatoxin biosynthetic gene cluster is partially elucidated. The sequences of some of the genes are already known and published (Trail et al., 1995b). Interestingly, the genes are organized in such a way that the gene encoding the first enzyme in the pathway is located at one end of the cluster and the other genes follow in the same order as the enzymatic reactions in the biosynthetic pathway (Fig. 11.1). Due to this arrangement, the gene for norsolorinic acid reductase (nor-1) is located at one end of the cluster, the gene for versicolorin A dehydrogenase (ver-1) lies in the middle, and the gene for sterigmatocystin-O-methyltransferase is located at the other end of the cluster. By selecting these genes as target sequences in a multiplex PCR, the whole aflatoxin biosynthetic gene cluster can be covered, and partial deletions of the cluster can be detected (Geisen, 1996). Conventional morphological methods for the detection of aflatoxinogenic fungi cannot distinguish between aflatoxin producing and non-producing strains. If the non-producing phenotype is due to a deletion of the biosynthetic gene cluster or a part thereof, or to nucleotide changes at the primer binding sites, the PCR approach is able to distinguish between both genetic alterations.

In a multiplex PCR system, several genes can be detected in one reaction at a time. Several primer sets are added to the reaction mixture and the reaction is carried out at an optimized temperature. After electrophoresis of
the products, several amplicons become visible. Multiplex PCR with up to 18 primers has been described (Chamberlain et al., 1989). The advantage of this system is improved specificity. It was observed that some secondary metabolite biosynthetic genes are more widely distributed in different fungal species than expected (see later for details). Thus, it might happen that a PCR product of the same length as the diagnostic PCR product may occur in a mycotoxin non-producing species. This situation reduces specificity drastically if only a monomeric PCR is used. The occurrence of two or more

![Fig. 11.1. Schematic illustration of the aflatoxin biosynthetic pathway. The order of the genes reflects their relative location in the aflatoxin biosynthetic gene cluster, according to Yu et al. (1995). The target genes of the multiplex PCR analysis are indicated in boxes.](image)

**Enzymes**
- Norsolorinic acid reductase
- Versicolorin A dehydrogenase
- Sterigmatocystin-O-methyltransferase

**Genes**
- pksA
- nor-1
- uvm8
- allR
- ver-1
- ord-1
- ord-2
- omtA

**Metabolites**
- Acetate
- Polyketide
- Norsolorinic acid
- Averantin
- Averufarin
- Averulin
- Hydroxyversicolorone
- Versicolonal hemiacetal acetate
- Versicolorin B
- Versicolorin A
- Sterigmatocystin
- O-methylsterigmatocystin
- Aflatoxin
PCR products of identical length as the diagnostic PCR products with template DNA of non-producing species, however, is unlikely. In addition, a multiplex reaction has the advantage that an internal control reaction can be included which signals the activity of the polymerase.

Due to the complexity of a multiplex PCR system, several requirements must be met. The reaction kinetics of the different primer sets should be similar to ensure that comparable amounts of PCR products are produced during a reaction. The reaction kinetics are strongly dependent on the primer design: G/C content, melting temperature, secondary structures, 5'3' overlap. A G/C content of 40–60% and a length of 23–28 nucleotides are suggested as general guidelines for specific annealing. The primary structure of published aflatoxin biosynthetic gene-specific primers are given in Table 11.3.

Another important aspect for the development of a multiplex reaction is the primer location. They should be chosen in such a way that the resulting PCR products are easily separated by agarose gel electrophoresis. The primer binding sites for the detection of aflatoxinogenic fungi with the multiplex PCR system are shown in Fig. 11.2. The presence of introns in two of the amplicons does not influence the result. This indicates that the intron position and length are very conserved in aflatoxin biosynthetic genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>aflR &gt;</td>
<td>TA1CTCCCCCCCCGCGATCCTCCCG</td>
<td>450</td>
<td>Shapiro et al. (1996)</td>
</tr>
<tr>
<td>aflR &lt;</td>
<td>CGTCAGACAGCAGCCTGGACACGG</td>
<td>1482</td>
<td></td>
</tr>
<tr>
<td>nor1 &gt;</td>
<td>ACCGCTACGCGGCACCTCGGCAC</td>
<td>501</td>
<td>Geisen (1996)</td>
</tr>
<tr>
<td>nor1 &lt;</td>
<td>GTGGCCGCAGCTCGACACTCC</td>
<td>900</td>
<td></td>
</tr>
<tr>
<td>ver1 &gt;</td>
<td>ATGTCCGATAATACCGTTAGATGGC</td>
<td>496</td>
<td>Shapiro et al. (1996)</td>
</tr>
<tr>
<td>ver1 &lt;</td>
<td>CAAAAGCGCCACATTCCACCCCAATG</td>
<td>1391</td>
<td></td>
</tr>
<tr>
<td>ver1 &gt;</td>
<td>GCCGGCGGCTTCTCTGGCTCTAACG</td>
<td>623</td>
<td>Geisen (1996)</td>
</tr>
<tr>
<td>ver1 &lt;</td>
<td>GGGGATAACTCCCCGCCACAGGCC</td>
<td>1160</td>
<td></td>
</tr>
<tr>
<td>omtA &gt;</td>
<td>GCCGGCCGATCCTCTGCTGCTTAG</td>
<td>208</td>
<td>Shapiro et al. (1996)</td>
</tr>
<tr>
<td>omtA &lt;</td>
<td>CCCCATTGAGACCCCTTCCTCG</td>
<td>1232</td>
<td></td>
</tr>
<tr>
<td>omtA &gt;</td>
<td>GTGGACCGGACCTACGCGACATCAC</td>
<td>301</td>
<td>Geisen (1996)</td>
</tr>
<tr>
<td>omtA &lt;</td>
<td>GTCCGGCCGCAGCAGCCTGGGTGGGG</td>
<td>1098</td>
<td></td>
</tr>
</tbody>
</table>

aThe sequences are given 5' to 3'.
bThe positions of the first and last nucleotide of the amplified product are given relative to the first codon of the gene.
The results of typical multiplex PCR analyses with DNA from aflatoxinogenic fungi are shown in Fig. 11.3. All aflatoxin-producing strains show the same triplet pattern after electrophoretic separation of the PCR products. The pattern of the aflatoxinogenic *A. flavus* strains was identical to that of the *A. parasiticus* strains, indicating the homology of the aflatoxin biosynthetic genes in both species, which is also described in the literature (Yu et al., 1995). Non-aflatoxinogenic *A. flavus* strains gave variable results. One strain showed no signal at all, indicating a complete or nearly complete deletion of the aflatoxin biosynthetic gene cluster. Another strain exhibited a doublet pattern, the band for the *omt-A* gene was missing, suggesting a deletion of the part of the gene cluster containing the genes for the last reactions in the biosynthetic pathway. A third strain possessed all three PCR DNA product bands, demonstrating another type of mutation, perhaps in one of the regulatory genes. Most of the non-aflatoxinogenic strains which were analysed showed changes in the triplet pattern, suggesting that their phenotype was due to deletions of the aflatoxin biosynthetic genes.

*A. versicolor*, which is not an aflatoxin-producing species, showed the same triplet pattern. This was not unexpected as *A. versicolor* is a sterigmatocystin producer, and sterigmatocystin is the precursor of aflatoxin (Dutton, 1988). It is synthesized as an end-product of a secondary metabolite pathway in some fungi like *A. versicolor*, *A. nidulans*, and in species of *Chaetomium*, *Monocillium*, *Farrowia* and *Bipolaris*. Sterigmatocystin by itself is an important mycotoxin, although with a reduced toxicity compared with aflatoxin (Feuell, 1969). The sterigmatocystin biosynthetic pathway is very similar to the one for the aflatoxin biosynthesis. For example, the genes for the conversion of versicolorin A to sterigmatocystin are very conserved in both pathways. In addition, *aflR*, a regulatory gene of the aflatoxin biosynthetic pathway, is able to induce the genes of the sterigmatocystin

![Diagram of primer binding sites](image)

**Fig. 11.2.** Illustration of the primer binding sites of the three aflatoxin biosynthetic gene-specific primer pairs used for multiplex PCR. Thick lines represent the aflatoxin biosynthetic gene coding sequences, cross-hatched boxes indicate intron sequences.
biosynthetic pathway when introduced into a mutant of *A. nidulans*. A gene for a polyketide synthase which is required for aflatoxin biosynthesis was also identified in *A. nidulans* (Brown et al., 1996).

As mentioned above, the method should differentiate between *A. flavus*, *A. parasiticus* and the related species, *A. oryzae* and *A. sojae*. Figure 11.3 shows that this is, indeed, the case. Both fermentation organisms obviously carry only a part of the aflatoxin biosynthetic gene cluster, as two bands specific for the *ver-1* and *omt-A* genes appear. But the band for the *nor-1* gene is missing, indicating a deletion of the beginning of the gene cluster. The method clearly differentiates between these two GRAS organisms and the aflatoxinogenic fungi. These organisms do not have the same habitat and *A. oryzae* and *A. sojae* are only scarcely found in nature. Both strains are adapted to a fermentative food environment, which is not the typical habitat.
for aflatoxinogenic species. They are, however, morphologically very similar to the aflatoxinogenic species and the ability of the PCR method to differentiate between these species can be used for the confirmation and safety control of the GRAS species used for production of human food.

This diagnostic PCR method gave negative results with many of the food-borne fungal species tested (Table 11.4). These included different species of the genera *Penicillium*, *Fusarium*, *Aspergillus*, *Byssochlamys* and *Geotrichum*. Two interesting exceptions were several strains of *Penicillium roqueforti* and *P. italicum*. Both species gave a positive signal in the case of the *nor-1* specific band. The PCR product which appeared had the same migration behaviour as the *nor-1*-specific amplicon from an aflatoxinogenic strain. To analyse potential sequence homology, a digoxigenin-labelled *nor-1* PCR product was used as a probe for hybridization to the PCR product from *P. roqueforti*. A weak signal indicated some homology, suggesting that *P. roqueforti* contains sequences with homology to the *nor-1* gene of aflatoxinogenic fungi. The results demonstrate the advantage of multiplex PCR analyses over monomeric reactions with regard to their specificity.

Shapiro et al. (1996) described a similar diagnostic PCR method for the detection of aflatoxinogenic fungi. Three target genes, the *omt-1* gene, the *ver-1* gene, and the *apa-2* gene, from the aflatoxin biosynthetic gene cluster were used in three separate monomeric PCRs. The *apa-2* (now named as *aflR*) gene is a regulatory gene, influencing the expression of the other genes.

### Table 11.4. Results of PCR analyses specific for aflatoxin biosynthetic genes with food-borne fungi.

<table>
<thead>
<tr>
<th>Species</th>
<th>BFE strain</th>
<th>nor-1</th>
<th>ver-1</th>
<th>omt-A</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Penicillium italicum</em></td>
<td>BFE45</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>P. digitatum</em></td>
<td>BFE46</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>P. nalgiovense</em></td>
<td>BFE66</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>P. camemberti</em></td>
<td>BFE135</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>P. chrysogenum</em></td>
<td>BFE236</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>P. roqueforti</em></td>
<td>BFE278</td>
<td>+</td>
<td>+&lt;sup&gt;c&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td><em>Byssochlamys nivea</em></td>
<td>BFE218</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Geotrichum candidum</em></td>
<td>BFE222</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Fusarium moniliforme</em></td>
<td>BFE322</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>F. graminearum</em></td>
<td>DSM1095&lt;sup&gt;b&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>DSM62376</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>DSM62416</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>F. sporotrichoides</em></td>
<td>DSM62423</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup>Strain number according the culture collection of the Bundesforschungsanstalt für Ernährung.

<sup>b</sup>Strain number of the German Culture Collection (DSM).

<sup>c</sup>The PCR fragment was slightly larger than that of *A. parasiticus*. 

Detection of Mycotoxin-producing Fungi
aflatoxin biosynthetic genes (Chang et al., 1993). In contrast to the results described by Geisen (1996), no strong positive PCR signals were found with DNA from aflatoxinogenic A. flavus strains. After 30 cycles a weak signal for the omt-1 gene appeared, and after 40 cycles, a weak band for the ver-1 gene became visible with DNA from A. flavus as the template, but a reaction with the aflR-specific primer pair failed. The sequences for the aflR genes of A. flavus and A. parasiticus are highly homologous, but show distinct differences according to Chang et al. (1995). These sequence dissimilarities might be responsible for the failure to detect the aflR gene in A. flavus by PCR. Shapiro et al. (1996) could not identify the omt-1 gene in A. nidulans, a potential sterigmatocystin-producing species, and they argued that no omt-1 gene was present. The PCR approach described by Geisen (1996) however, demonstrates that at least in A. versicolor, an omt-1-homologous sequence was present.

Detection of aflatoxinogenic A. flavus strains in a food system

Aflatoxinogenic fungi are found predominantly in certain foods such as peanuts, almonds, figs and spices (Bullermann, 1979). Figs are often infected by members of the group of black aspergilli, including A. flavus and A. parasiticus (Doster et al., 1996). Figs that were infected with an aflatoxinogenic strain of A. flavus could be identified with the PCR approach. The expected amplicons were generated with DNA isolated from infected figs, whereas uninfected figs gave no signal at all (Färber et al., 1997).

The most crucial prerequisite for diagnostic PCR with food samples is the isolation of sample DNA in a PCR-usable form. It is known that certain food components like fats and proteins can interfere with the activity of Taq polymerase (Rossen et al., 1992). An inhibition of the PCR was also observed for the infected figs, indicating that a high concentration of carbohydrates is also inhibitory to the PCR system. The sample DNA was prepared by a phenol extraction method. An estimation of the sensitivity of the reaction revealed that it was reduced by a factor of ten compared with pure fungal DNA.

Kreutzinger et al. (1996) described a method for reducing inhibitory activity in PCR from environmental samples. In an attempt to detect ectomycorrhizal fungi in their natural habitat by PCR, they observed that the Taq polymerase was inhibited strongly by compounds in the sample. This problem was overcome by the use of very dilute template DNA in a first round of PCR, followed by a second reaction with nested PCR primers. Lantz et al. (1994) have described another approach for the preparation of sample DNA. They attempted to detect pathogenic Listeria monocytogenes cells in soft cheese by PCR. However, with conventional sample DNA preparation protocols, the reaction failed; foods with a high fat content are particularly inhibitory to the polymerase reaction. Functional template DNA can be prepared if an aqueous, two phase sample preparation system is used. With this system, the sample is homogenized in a mixture of two
non-soluble aqueous solutions, which separate in two phases after homoge-
nization. One phase consists of a polyethylene glycol (PEG) solution and the
other of a dextran solution. This extraction system separates PCR inhibitory
compounds, which partition to the PEG phase. If samples from the dextran
phase are used for PCR, \textit{L. monocytogenes} cells can be detected.

Shapiro \textit{et al.} (1996) used their PCR method for the detection of
aflatoxinogenic fungi in corn. They described an enrichment procedure to
amplify the template DNA prior to PCR by suspending the corn sample in
a rich medium; the sensitivity of the method increased with the incubation
time but the detection time increased simultaneously. Only corn inoculated
with aflatoxinogenic \textit{A. parasiticus} gave positive signals with the PCR and
after 24 h incubation, $1 \times 10^2$ spores of \textit{A. parasiticus} could be detected.

Even with sophisticated DNA preparation protocols, the occurrence of
false negative reactions with food samples remains a problem. The use of an
internal standard can give information about the quality of the reaction. If a
universal primer set is used which gives a PCR product with all food-relevant
fungal species, then a DNA band of a particular length should occur in nearly
all food samples where there is contaminating fungal biomass. Alternatively,
the sample can be spiked with purified fungal DNA of a certain species. The
occurrence of the standard amplicon is then an indication of the efficiency of
the reaction. It is advantageous to use a universal primer set instead of a
specific one, as every available fungal DNA can then be used as an internal
standard. A universal fungus-specific primer system has been described by
Kappe \textit{et al.} (1996); the target sequences of this system are the 17S ribosomal
RNA genes. Similarly, the conserved nucleotide sequences of the rRNA
genes, which can serve as primer sequences for the amplification of the ITS1
and ITS2 regions, have been described (White \textit{et al.} 1990).

The primers ITS1 and ITS2 can be added as a fourth primer pair to the
multiplex PCR. They amplify the ITS1 region from different fungal species
and the PCR product is approximately 250 bp long, although the length may
vary depending on the template DNA used. Figure 11.4 shows some typical
results. The aflatoxinogenic strain now shows a quadruplet pattern as
expected. All other strains, including different \textit{Penicillium} or \textit{Aspergillus}
species, show only the standard band of the ITS1 region. As a result of this
reaction design, a sample with a quadruplet pattern indicates the presence of
an aflatoxinogenic species, whereas a monomeric band of about 250 bp
indicates that the reaction was not inhibited by food components and that the
sample did not contain potential aflatoxinogenic fungi.

11.2.2 \textbf{PR toxin producing fungi}

\textit{Penicillium roqueforti} occurs in various natural habitats. It is used as a fungal
starter culture for the production of blue-veined cheese (Marth, 1987), but
can also be found as a spoilage organism in different food and feed
commodities (Frisvad, 1988). \textit{P. roqueforti} is a heterogeneous species that
was recently divided, according to secondary metabolite patterns and differences in its ITS regions, into three distinct species: *P. roqueforti*, *P. paneum* and *P. carneum* (Boysen et al., 1996). All three species are able to produce various secondary metabolites: *P. carneum* is capable of producing patulin, penitrem A and mycophenolic acid; *P. roqueforti* produces PR toxin, marcfortines and fumigaclavine A; and *P. paneum* produces patulin, botryodiplidin and other secondary metabolites. All three species are morphologically very similar. *P. roqueforti* is the species which is used exclusively for the production of blue cheese. All strains isolated from blue cheese are able to produce PR toxin (Geisen and Holzapfel, 1995). PR toxin is a relatively toxic substance, which is able to inhibit protein translation in eukaryotic cells (Moule et al., 1978). However, PR toxin is not stable in the cheese matrix where it is converted to the much less toxic PR imine (Scott, 1981). Nevertheless, strains which do not produce PR toxin are preferable over

Fig. 11.4. Agarose gel of the multiplex PCR products of different food-borne fungi. Chromosomal DNA of these strains was isolated and subjected to PCR analysis using the aflatoxin biosynthetic gene-specific primers in addition to a primer pair specific for the ITS1 region of the rRNA genes. Lane 1, *F. moniliforme* BFE312; lane 2, *F. solani* BFE325; lane 3, *P. digitatum* BFE350; lane 4, *A. flavus* BFE312 (aflatoxin negative); lane 5, *A. flavus* BFE84 (aflatoxin positive); lane 6, size standard, fragment sizes are indicated in bp.
conventional strains with respect to safety considerations. PR toxin is a sesquiterpenoid secondary metabolite, with acetyl-CoA as the first precursor. One of the key enzymes in its biosynthetic pathway is the aristolochen synthase, a sesquiterpene cyclase. The nucleotide sequence of this gene is known (Procter and Hohn, 1993) and, by using gene-specific PCR with the aristolochen synthase gene (ari1) as a target, it is possible to screen P. roqueforti strains for the presence of aristolochen synthase. Strains with a deletion in that gene are expected to be unable to produce PR toxin. This method of screening for PR toxin-free strains is more direct, reliable and less time-consuming than looking for the ability to produce PR toxin especially as PR toxin production is dependent on various environmental conditions (Scott et al., 1977), which makes the identification of PR toxin-negative strains by conventional methods difficult.

Strains isolated from cheese and infected feeds were subjected to PCR analysis with ari1-specific primers. A product of the expected length occurred in nearly all strains, indicating the presence of the ari1 gene. Figure 11.5 shows a typical result obtained with several strains. Subsequent analysis by thin layer chromatography (TLC) showed that all strains except one produced PR toxin. The strains which were negative in the PCR, but which were able to produce PR toxin, were verified by dot-blot hybridization using an ari1-specific probe. After the analysis all PR toxin-producing strains were positive either in the dot-blot hybridization or the PCR analysis (Table 11.5). An exception was one strain which did not produce PR toxin, indicating that it did not carry the ari1 gene. The other strains which were negative in the PCR analysis, but positive in the dot-blot hybridization and TLC obviously carried active genes which may have alterations in their primer binding sites. These results show that it is not always appropriate to rely solely on one PCR target site, as small changes in the sequence can have dramatic effects on the PCR results. The single strain which was negative in the PCR analysis and did not produce PR toxin, was originally isolated from cheese and is therefore probably a safe candidate for the production of blue-veined cheese.

As mentioned above, homologues of aflatoxin biosynthetic genes can be identified unexpectedly in P. roqueforti (Table 11.5). The same phenomenon is apparent with the ari1 gene and a sequence homologous to that gene can be found unexpectedly in various species (Table 11.6). The gene-specific PCR gave positive results with P. camemberti and Byssochlamys nivea, both species which are not known to produce PR toxin. With the dot-blot analysis even more species gave positive results, indicating the occurrence of ari1-like sequences in a variety of species, which are not able to produce PR toxin. The relevance of the frequent occurrence of a nucleotide sequence homologous to secondary metabolite biosynthetic genes in various fungi is not known. Enzymes of secondary metabolite pathways have less specificity than their counterparts from primary metabolism (Dutton, 1988) and this results in a somewhat different behaviour from that of biosynthetic enzymes of primary metabolism. In certain cases, one enzyme can catalyse analogous reactions
with related but different substrates. This can result in a metabolic grid, a network of enzymatic reactions from different substrates that give rise to different reaction products (Yabe et al., 1991). This is one of the reasons why in secondary metabolism a large number of end-products can be produced, such as the aflatoxins, the fumonisins, the trichothecens or the ochratoxins. This behaviour may also be an explanation for the positive PCR results for specific secondary metabolite biosynthetic genes in various species. If these species possess active genes with homology to mycotoxin biosynthetic genes,
their gene products may participate in secondary metabolite pathways leading to different, perhaps as yet unidentified, end-products.

These results taken together suggest that a monomeric PCR reaction, which targets only one gene, possesses insufficient specificity for the detection of mycotoxin-producing fungi. Multiplex reactions, as was described for aflatoxinogenic fungi, are recommended. If only one gene of the mycotoxin biosynthetic pathway is known, as in the case of PR toxin, another known specific sequence can be used as an additional target sequence, for example the sequences of the rRNA genes of \emph{P. roqueforti} (Boysen et al., 1996).

<table>
<thead>
<tr>
<th>BFE strain$^a$</th>
<th>arl$^b$</th>
<th>Dot-blot$^c$</th>
<th>PR toxin$^d$</th>
<th>nor l$^e$</th>
<th>Source$^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFE42</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>C</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>C</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>ND</td>
<td>C</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>–</td>
<td>C</td>
</tr>
<tr>
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<td>+</td>
<td>ND</td>
<td>C</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>ND</td>
<td>C</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
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<td>C</td>
</tr>
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<td>BFE50</td>
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<td>+</td>
<td>+</td>
<td>–</td>
<td>C</td>
</tr>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>C</td>
</tr>
<tr>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>C</td>
</tr>
<tr>
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<td>+</td>
<td>C</td>
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<tr>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>S</td>
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<tr>
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<td>+</td>
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<td>+</td>
<td>ND</td>
<td>S</td>
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<tr>
<td>BFE211</td>
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<td>+</td>
<td>ND</td>
<td>S</td>
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<tr>
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<td>+</td>
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<td>S</td>
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<tr>
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<td>S</td>
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<tr>
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<td>S</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
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$^a$Strain number according the culture collection of the Bundesforschungsanstalt für Ernährung.
$^b$The presence of the arl gene sequences was checked with gene-specific PCR.
$^c$For dot-blot analysis the labelled arl-specific PCR product was used.
$^d$Detected by thin layer chromatography.
$^e$The presence of the nor l gene was checked by gene-specific PCR.
$^f$C, isolated from cheese; S, isolated from silage.
ND, not determined.
11.2.3 rDNA and miscellaneous sequences as target sequences

The organization of ribosomal RNA genes is highly conserved within the fungal kingdom. The small subunit, 5.8S, and large subunit rRNA genes are organized in a single transcription unit and are separated by two spacer regions ITS1 and ITS2. The RNA transcript is post-transcriptionally processed into small subunit, 5.8S, and large subunit rRNA species. The rRNA sequences have conserved and variable regions and are useful for studying distantly related organisms at the taxonomic level. The ITS sequences, however, are more variable in their nucleotide composition and can be used for differentiation of species or populations. It may be possible to identify unique sequences within the ITS regions which may serve as target sequences for diagnostic PCR. Universal primers which can be used for amplification or cycle sequencing of ITS1 and ITS2 have been described (White et al., 1990; Fig. 11.6). It is relatively straightforward to sequence these regions by using primers such as ITS1 and ITS4, and to determine unique sequences for primer development. A prerequisite for usage of these sequences for detection of mycotoxinogenic fungi is the requirement that their presence be linked to the ability of a strain to produce a mycotoxin. This is one of the drawbacks of this approach as in most cases not all strains of a species are able to produce the respective mycotoxin. A species-specific primer pair identified by this approach does not ultimately mean that it is

### Table 11.6

Presence of aristolochen synthase gene like nucleotide sequences in various species.

<table>
<thead>
<tr>
<th>BFE strain</th>
<th>Detection of <em>ari1</em>-like sequences by PCR</th>
<th>Detection of <em>ari1</em>-like sequences by Dot-blot</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. roqueforti</em> BFE278</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. camemberti</em> BFE135</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. nivea</em> BFE218</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>P. italicum</em> BFE45</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>P. naRioverse</em> BFE66</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>A. nidulans</em> BFE125</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>P. digitatum</em> BFE46</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>P. chrysogenum</em> BFE236</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>G. candidum</em> BFE222</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>A. flavus</em> BFE388</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>A. parasiticus</em> BFE293</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>A. versicolor</em> BFE294</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*aStrain number according the culture collection of the Bundesforschungsanstalt für Ernährung.*

### Table 11.6

Presence of aristolochen synthase gene like nucleotide sequences in various species.
specific only for the mycotoxinogenic strains of that species. A positive result from PCR of the rRNA genes is, therefore, not as valid as the results from a PCR targeted to the mycotoxin biosynthetic genes.

Considerable literature data on ITS sequences of different fungi are available. Most of the sequences were obtained for taxonomic purposes and their specificity in PCR analyses has not been determined (Morales et al., 1993; Bunting, et al., 1996; Waalwijk et al., 1996). F. sambucinum (teleomorph, Gibberella pulicaris) is able to produce fusarin C and trichothecenes (Smith and Solomons, 1994). O'Donnel (1997) has described three different groups of ITS sequences within the species F. sambucinum. However, the possible relationship between these ITS groups and the ability to produce a mycotoxin was not considered. Grimm and Geisen (1996) sequenced the ITS1 region of fumonisin-producing Fusarium species. They were able to identify regions which were common to all fumonisin-producing fungi, but which showed minor polymorphisms in sequence compared with non-fumonisin-producing species. On the basis of these differences, primer pairs were derived which gave only the expected products from F. moniliforme, F. nygamai, F. napiforme and F. proliferatum, i.e. potential fumonisin-producing fungi.

Schilling et al. (1996) analysed the ITS region of Fusarium culmorum (teleomorph, unknown), F. graminearum (teleomorph, Gibberella zeae) and F. avenaceum (teleomorph, G. avenacea) with the aim of developing ITS-specific PCR primers. All three species are plant pathogens of cereals and grasses. The three species are able to produce different mycotoxins: F. avenaceum produces moniliformin and trichothecene A; F. graminearum produces zearelenone, trichothecene A + B and fusarin C; and F. culmorum produces zearelenone, trichothecene A + B and culmorin. Schilling et al. (1996) were not able to identify sufficient sequence polymorphisms within the ITS region of these three species to identify specific primer target sites. For this reason, they applied another approach in which preselected random sequences were chosen as target sequences for the PCR primers. This preselection was achieved by identifying RAPD–PCR patterns which were specific for each species. RAPD–PCR is a variation of conventional PCR (Williams, 1990) in which one primer of arbitrary sequence is used. The hybridization temperature during RAPD–PCR is considerably reduced compared with conventional PCR. This enables semi-specific binding of the
primer and results in a specific RAPD pattern. If identical conditions are used, the same pattern should be generated with each reaction. Depending on the type of random primer used, the resulting RAPD pattern can be species-specific (Guthrie et al., 1992), and can differentiate between various genotypes within a species (Hamelin et al., 1993) or even between strains of the same species (Bidochka et al., 1994). The probability that certain bands of the RAPD pattern which occur only in mycotoxin-producing fungi consists of unique sequences is very high. Schilling et al. (1996) isolated unique bands of the RAPD pattern of Fusarium species. They derived specific PCR primer target sites from their sequences which could be used for differentiation and detection of these species.

The approach described above may be a general method for the development of a diagnostic PCR method for a particular group of microorganisms if no genetic data are available. The same approach was used for the development of gene probes for potential fumonisin-producing fungi (Geisen, 1997). Two RAPD bands were identified which only occurred with potential fumonisin-producing fungi, such as F. moniliforme, F. nygamai, F. napi forme and F. proliferatum. These two DNA fragments were labelled either with digoxigenin or with biotin and used as probes for various fungal DNA sequences. Specific hybridization of the probes was visualized by alkaline phosphatase reactions with either fast blue B (resulting in a blue colour) or fast red TR (resulting in a red colour). When both colours were present on the membrane the result was interpreted as the presence of a potential fumonisin-producing fungus. A survey of the results with the DNA from various food-borne fungi is given in Table 11.7. From these results it can be concluded that primer sequences for diagnostic PCR can be derived from RAPD fragments as also shown by Schilling et al. (1996).

In addition to rRNA genes or random sequences, sequences from cloned genes of mycotoxin-producing fungi may serve as targets for diagnostic PCR. Niessen and Vogel (1997) developed a PCR analysis which was specific for F. graminearum. All other Fusarium species or species of other genera gave negative results, indicating high specificity for the method. The reaction was directed against the endogenous galactose oxidase gene (gaoA); however, the authors did not attempt to correlate mycotoxin production and PCR results.

11.3 Conclusions

PCR is a valuable tool for the rapid screening of foods and feeds for the presence of mycotoxin-producing fungi. Results can be obtained from an infected sample within 24 h as compared with up to several days by conventional methods. If an appropriate target sequence is chosen, the method can also differentiate between mycotoxin-producing and non-producing strains of a species in most cases.
Problems with inhibitory substances within food samples can be overcome by an adapted DNA purification protocol (Lantz et al., 1994), the use of highly diluted DNA and a subsequent nested second amplification (Kreutzinger et al., 1996), or controlled by the use of internal standard DNA giving information about the quality of the reaction.

The most direct procedure for the development of a diagnostic PCR method for mycotoxin-producing fungi is the targeting of the mycotoxin biosynthetic genes. If their sequences are not available, other opportunities for the identification of target sequences exist, such as the determination of specificities in ITS sequences, or random sequences preselected by RAPDs; sequences from structural genes may also serve as target sites. These secondary target sequences are less specific because mycotoxin-producing and non-producing strains are indistinguishable. Diagnostic PCR methods

| Table 11.7. Dot-blot analysis with the RAPD gene probes for fumonisin-producing fungi. |
|------------------------------------------|-------------------|-------------------|
| BFE straina                          | Probe 1 | Probe 2 |
| *Penicillium italicum* BFE45           | –       | –     |
| *P. digitatum* BFE46                   | –       | –     |
| *P. raigiovente* BFE66                 | –       | –     |
| *Aspergillus flavus* BFE84             | –       | +     |
| *A. nidulans* BFE125                   | –       | –     |
| *P. camemberti* BFE125                 | –       | –     |
| *Byssochlamys nivea* BFE218             | –       | –     |
| *Fusarium moniliforme* BFE314          | +       | +     |
| *F. moniliforme* BFE315                 | +       | +     |
| *F. moniliforme* BFE316                 | +       | +     |
| *F. moniliforme* BFE317                 | +       | +     |
| *F. moniliforme* BFE318                 | +       | +     |
| *F. moniliforme* BFE319                 | n.d.    | +     |
| *F. moniliforme* BFE320                 | +       | +     |
| *F. proliferatum* BFE343               | +       | +     |
| *F. proliferatum* BFE344               | +       | +     |
| *F. nygama* BFE353                      | +       | +     |
| *F. nygama* BFE355                      | +       | +     |
| *F. nuygama* BFE354                     | +       | +     |
| *F. graminearum* BFE323                 | –       | –     |
| *F. poae* BFE324                        | –       | –     |
| *F. solani* BFE325                      | –       | –     |
| *F. sporotrichoides* BFE326             | –       | –     |

*aStrain number according the culture collection of the Bundesforschungsanstalt f¨ur Ern¨ahrung.
nd not determined.

Detection of Mycotoxin-producing Fungi
are rapid tools for safety control of foods and feeds. They can, however, only give information about the presence of a potential mycotoxin-producing fungus in a sample but cannot detect the mycotoxin itself. A positive result indicates a potential health hazard and the product should be further checked for the presence of the particular mycotoxin by analytical methods.

Acknowledgements

P. Thiel and J. P. Rheeder are thanked for providing fumonisin-producing *Fusarium* species, and H. Auerbach for the *P. roqueforti* strains from silage.

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divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (Gibberella pulicaris). Current Genetics 22, 213–220.


12.1 Introduction

Recent advances in the use of immunosuppressive therapies to treat cancer and enable solid organ or bone marrow transplants, together with advances in the development of broad spectrum antibiotics, have created an increasing population of immunocompromised patients. In addition, the HIV pandemic has created a large increase in the number of immunosuppressed individuals. These patients are at significant risk from systemic fungal infections. The incidence of invasive fungal infection in bone marrow transplant patients has been reported to be as high as 50%, and the subsequent mortality rates are generally around 80% (Tang and Cohen, 1992). The three major opportunistic fungal pathogens in the UK are *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans* but many other fungi have also been reported as pathogens in these clinical settings. These include non-*albicans* *Candida* spp., especially *Candida krusei* and *Candida glabrata*, *Aspergillus flavus*, *Pneumocystis carinii*, *Fusarium* spp., *Trichosporon beigelii*, *Rhizopus arrhizus*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis* and *Coccioidoides immitis* (Bodey, 1988; Anaissie et al., 1989). With the exception of the cryptococcal antigen latex agglutination test, established methods for diagnosing systemic fungal infections are often time-consuming and insensitive; e.g. blood cultures are nearly always negative in cases of invasive aspergillosis (Richardson and Warnock, 1993). If appropriate therapy is to improve the prognosis of the immunocompromised patient with systemic fungal infection, early diagnosis is required (Burch et al., 1987).

Antibody responses in immunocompromised hosts are generally too weak to form the basis of a diagnostic test. However, a large research
investment has been made in the development of antigen detection-based immunoassays for the diagnosis of various systemic mycoses including both invasive candidosis and aspergillosis, disseminated histoplasmosis, coccidioidomycosis and paracoccidioidomycosis. While these tests have the advantages of being both quick and relatively straightforward to perform, they have not gained general acceptance due to low sensitivity and/or lack of commercial availability (De Repentigny et al., 1994). Extracellular galactomannan (GM) circulating in patients with invasive aspergillosis can be detected in serum samples with a commercially available latex agglutination test (Pastorex Aspergillus, Sanofi Diagnostics Pasteur, Marnes-La Coquette, France) which uses a monoclonal antibody (raised against A. fumigatus GM) to detect antigen. However, reports on the utility of this diagnostic test vary (Van Cutsem et al., 1990; Kappe and Schulze-Berge, 1993; Haynes and Rogers, 1994; Manso et al., 1994; Verweij et al., 1995). In contrast with other fungal infections, diagnosis of cryptococcosis by the cryptococcal antigen latex agglutination test is accepted as the standard method of diagnosis, with a high sensitivity and specificity. Most equivalent tests are not sensitive enough to exclude diagnosis on the basis of a negative result.

Commercial biochemical tests are available for identifying clinical yeast isolates, e.g. Vitek Yeast Biochemical Card (bioMerieux Vitek Inc., Hazelwood, Missouri) and the API 20C identification system (bioMerieux Vitek Inc.); both are reliable for the identification of common yeasts but problems of non-specificity have been encountered with more unusual species (Fenn et al., 1994).

Inability or delay in diagnosing fungal infection defers the administration of appropriate therapy. This has grave implications for the prognosis of the patient: reliable and rapid diagnostic tests for systemic mycoses are imperative to improve rates of patient survival (Haynes and Rogers, 1996).

In attempting to overcome low sensitivity, poor specificity and delay, molecular approaches to the diagnosis of fungal infection have been adopted, particularly the polymerase chain reaction (PCR). PCR can be very sensitive and, depending on primer design, very specific. Several species-specific PCR-based diagnostic methods for detection of various common fungal pathogens have been described. This discussion will concentrate solely on the diagnosis of systemic fungal infection; however, many of the principles described could easily be adapted to the diagnosis of superficial or subcutaneous mycoses.

12.2 Strategies for Designing PCR-based Diagnostics

When developing a PCR-based diagnostic method various factors can be adapted to optimize both sensitivity and specificity.
12.2.1 Target

The specificity of a diagnostic PCR can be engineered to suit the purpose for which it is being used by selecting an appropriate target sequence for amplification. The specificity is determined by the degree of homology shared between the target sequence and DNA sequences of different genera or species. For example, a highly conserved region from a gene universally shared by all fungi is a suitable target sequence for a PCR protocol designed to indicate whether an infection is bacterial or fungal in origin. This strategy has been adopted by several groups who have selected highly conserved, or ‘universal’, regions within fungal ribosomal RNA (rRNA) genes as target sequences for amplification. Various amplicon detection methods can be used to identify the fungal source of DNA to genus or species level. The rRNA genes are a popular choice for this strategy as they contain highly conserved regions which can be used to amplify all DNAs, adjacent to highly variable regions which can be used for subsequent species identification. Another advantage is that rRNA genes are generally present at a high copy number which increases the sensitivity of the PCR. Makimura’s group (1994) developed a PCR based on conserved regions within the fungal 18S rRNA genes which successfully amplified DNA from 78 strains of 25 medically important fungi. Haynes et al. (1995) described a PCR in which one pair of primers corresponded to the V3 region of the S. cerevisiae large subunit rRNA gene sequences universally conserved within the fungal kingdom. Species-specific forward primers were then designed which could be used in tandem with the universal reverse primer to specifically amplify DNA from A. fumigatus, Cr. neoformans and C. albicans.

Hopfer et al. (1993) used a previously described set of primers based on an rRNA gene sequence present throughout the fungal kingdom (White et al., 1990) to amplify a 306–311 bp target in 42 different fungi. The amplicons were subjected to restriction enzyme analysis; PCR products were digested with HaeIII and separated by electrophoresis through agarose gels. The resulting patterns were used to place the sources of fungal DNA into five broad groups including Candida spp. and related yeasts, and Aspergillus spp. plus related septate moulds.

Alternatively, a highly variable region would give greater specificity, enabling the detection of a particular genus or species. Generally, the more variable the target region the more discriminatory the PCR (Mitchell et al., 1994b). Tang et al. (1993) described a PCR to detect the alkaline protease genes of A. fumigatus and A. flavus; the species were distinguished by the size of the amplified fragment. This PCR was shown to detect both A. fumigatus and A. flavus in four bronchoalveolar lavage (BAL) samples of four patients with either proven or possible invasive pulmonary aspergillosis; only one of 18 BAL samples from immunosuppressed patients with no clinical indication of fungal infection was positive by PCR.
12.2.2 Sample choice and preparation

As part of the developmental process for a diagnostic PCR protocol, the choice of clinical material and the method of DNA extraction from that material is critical. Ideally, the presence of target DNA in the chosen specimen will be clinically significant; the detection of *Candida* must distinguish pathogen from commensals, the detection of *Aspergillus* must distinguish pathogen from colonization or environmental contamination. Most PCR protocols for the diagnosis of *Candida* infections involve the examination of blood samples as detection of the fungus in blood is regarded as clinically significant. The diagnosis of invasive aspergillosis is more complicated as the fungus is rarely isolated from blood cultures and a reproducible PCR method for detecting *Aspergillus* DNA in blood remains elusive. This problem is complicated further as the significance of detection of this ubiquitous organism in respiratory specimens is questionable (see Section 12.3.2). Often a positive PCR result will need to be interpreted within the clinical setting to determine its relevance. As so many patients with systemic or pulmonary fungal infections are immunocompromised, the invasiveness of the procedure required to obtain the specimen must also be considered, particularly where sequential samples may be required.

Potentially suitable specimens for the diagnosis of systemic fungal infection are blood and/or serum, particularly as these may be obtained by relatively non-invasive procedures. The organism load may be very low or transient and a 5 ml blood sample is unlikely to be fully representative of the body’s 4–5 l. Therefore in order to decrease sampling error as much as possible, consecutive samples may need to be taken over a short period of time.

The DNA extraction method used must achieve two particular aims: to efficiently purify fungal DNA with minimal loss, and to remove inhibitors of PCR from the sample, e.g. haem. Breaking open the fungal cell wall is difficult but can be achieved either enzymatically or mechanically. Digestion with lytic enzymes such as Zymolyase (ICN Biomedicals Ltd, Thame) or NovoZym 234 (Calbiochem-Novabiochem Ltd, Nottingham) can aid DNA extraction, although mechanical means such as breaking open cells with glass beads have been successful, particularly for penetrating the thick polysaccharide capsule of *C. neoformans* (Tanaka *et al.*, 1996). Yields of extracted DNA may also be improved by using commercially available kits to purify DNA from proteins released by cell lysis (e.g. QIAamp Tissue Kit, Qiagen, Crawley). They are usually quicker to perform than established methods and avoid the use of phenol and chloroform, but they do add significantly to the cost of protocols.

12.2.3 Amplicon detection and analysis

The method used to detect or display amplicons resulting from PCR can increase both sensitivity and specificity. The simplest method is ethidium...
bromide staining of PCR products separated by electrophoresis through agarose gels. This has a detection limit of approximately 20 ng per amplified band, and in addition yields information on the size of the PCR product. An increase in sensitivity can be achieved if a nested PCR is performed. Yamakami et al. (1996) designed a nested PCR to detect Aspergillus species in serum of patients with invasive aspergillosis; the second (nested) round of PCR increased the sensitivity of detection by gel staining from 50 pg to 50 fg target DNA. Southern analysis increased the sensitivity another ten-fold to 5 fg.

Specific oligonucleotide probes have been developed for identification of PCR products from individual fungal species by Southern hybridization. Sandhu et al. (1995) used PCR to amplify a conserved region within the 28S rRNA genes from 50 fungal isolates (including common pathogens and saprophytes). Variable regions within 21 of these 50 isolates were used to design species-specific probes. When labelled with ³²P each probe hybridized only to homologous DNA. In order to avoid the use of radioisotopes, fluorescein can be used to label probes; fluorescein labelling of an internal probe followed by chemiluminescence (ECL detection system) of Southern blots allowed the confirmation of products from a PCR that amplified a region within the 18S rRNA gene universally conserved in all fungi (Polanco et al., 1995).

Alternatively, probes can be used as part of an enzyme immunoassay detection system. Species-specific probes are bound to the wells of a microtitre plate and denatured PCR products labelled with digoxigenin are hybridized to the probes; the products are visualized using anti-digoxigenin antibody conjugated to horseradish peroxidase with an appropriate chromogen substrate system, e.g. 3,3′,5,5′-tetramethylbenzidine (Sigma, Poole) and hydrogen peroxide. A colour reaction indicates the presence of target DNA identified by the specific probe. Probes are often captured on to the well surface by biotin-labelling the probe and precoating the wells with streptavidin, but dry-adsorption (evaporating a probe solution in the wells overnight at 37°C and heating for 2 h at 60°C) has been reported to be more efficient and negates the need for biotinylating probes (Hirayama et al., 1996). Fujita et al. (1995) described a PCR-immunoassay that generated amplicons with universal primers based on the internal transcribed spacer (ITS) region of fungal DNA; species-specific probes derived from this region were used to identify amplicons from C. albicans, C. tropicalis, C. parapsilosis and C. krusei. Using the biotin–avidin capture system and the C. albicans-specific probe, a detection sensitivity of two yeast cells per 0.2 ml blood was achieved.

Another approach to amplicon analysis is to look at single-strand conformational polymorphism (SSCP). Minor sequence changes in highly conserved regions of DNA will cause subtle morphological changes in DNA tertiary structure resulting in changes in a fragment’s mobility. Differences of only one base pair can be resolved by separating the DNA products
electrophoretically under non-denaturing conditions on a high-resolution acrylamide gel, allowing discrimination between species or even strains (Yap and McGee, 1994). Walsh et al. (1995) developed a universal PCR based on the 18S rRNA gene combined with SSCP detection to identify a variety of opportunistic fungal pathogens. The SSCP gel patterns enabled discrimination between Candida spp., Aspergillus spp., Cr. neoformans, Pseudallescheria boydii and R. arrhizus.

12.3 Diagnosis of Systemic Fungal Infections by PCR

Much research investment has been made into the development of PCR as a diagnostic tool in the field of medical mycology. The major developments in PCR-based diagnosis of invasive candidosis, invasive aspergillosis, P. carinii pneumonia, cryptococcosis and infections due to dimorphic fungi are discussed below.

12.3.1 Invasive candidosis

Candida spp., C. albicans in particular, are the most common fungal pathogens of immunocompromised hosts, causing a variety of infections from cutaneous disease of skin and nails to deep-seated systemic infection and candidaemia. Diagnosis is relatively straightforward for superficial infections but remains a problem for the more invasive forms of disease (Dupont, 1990). While most attention was initially paid to developing detection methods for C. albicans, as the primary pathogen of the genus, it has become apparent that other species of the genus may also cause disease. In a retrospective study of blood culture isolates from cancer patients approximately half (28 of 55) of the Candida isolates were C. albicans while the rest comprised of C. tropicalis (seven), C. glabrata (seven), C. krusei (five), C. parapsilosis (three), C. guilliermondii (three) and two mixed infections of C. albicans and C. glabrata (Meunier et al., 1992). Many of the PCR protocols published therefore describe strategies to detect both C. albicans and other species.

One of the first PCR methods for diagnosing fungal infection to be published was that by Buchman et al. (1990); the gene coding for Candida spp. cytochrome P₄₅₀ lanosterol-14α-demethylase (L1A1) was chosen as the target for amplification due to its involvement in the process of ergosterol biosynthesis. The sensitivity of the PCR was not fully assessed but was estimated in spiking experiments to be approximately 10²–10³ c.f.u. ml⁻¹. Clinical samples including urine, sputum, wound fluid and blood from six patients that had all undergone surgical trauma were tested by PCR; 15 of these gave positive results. However, the specificity of this PCR was not evaluated and the implications of the PCR-positive clinical samples was not discussed.
A sequence within a duplicated region of *C. albicans* mitochondrial DNA (mt DNA), EO3, was chosen by Miyakawa et al. (1992) as the target for a *C. albicans*-specific PCR. Primers, designed from partial sequencing of the region, generated a 1.8 kb fragment from 40 strains of *C. albicans* (type A and B) but not from any other of 38 isolates including seven non-*albicans* *Candida* spp., *Cr. neoformans*, *Saccharomyces cerevisiae*, three bacterial isolates and a human cell line. The sensitivity of the PCR was determined by spiking serial dilutions of yeast cells into both saline and human urine; the detection limits were two to ten cells and 100 cells in saline and urine respectively on ethidium bromide-stained gels. This was improved to two to ten cells for both saline and urine by Southern hybridization analysis. The authors conceded that such a large amplicon may be impractical when trying to detect small amounts of DNA present in clinical specimens such as blood, but suggested that smaller *C. albicans*-specific fragments (400–500 bp) within EO3 may amplify more efficiently.

Niesters et al. (1993) used a nested PCR in conjunction with a number of detection methods to identify various species of *Candida*. Sequences from the small subunit (SSU) rRNA genes of *C. albicans*, *C. glabrata* and *S. cerevisiae* were used to design primers which amplified the entire gene. Products were also obtained from *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *C. pseudotropicalis*, *C. stellatoidea* and *C. tropicalis*. Species-specific fingerprints generated by direct sequencing, Southern hybridization with species-specific probes, restriction enzyme mapping and interrepeat PCR (with repeat-oriented primers) were all able to identify these eight *Candida* isolates to species level. Restriction mapping provided the most rapid means of identification (1 day), but none of the methods were used to test clinical material.

In contrast to the high copy number gene targets described above, a PCR for diagnosing candidaemia was reported based on amplification of a 158 bp segment from the *C. albicans* actin gene (Kan, 1993). Amplicons were hybridized in the PCR reaction tube with a radiolabelled 30 bp internal probe before electrophoresis on acrylamide gels which were then exposed to autoradiography film. The sensitivity was reported as 25 fg of DNA or ten yeast cells in spiked samples. The specificity testing showed the PCR to be genus-specific, with the exception of *S. cerevisiae*. Preliminary evaluation of the PCR was carried out using immunocompetent mouse models and patient samples. Blood samples from a murine candidaemia model were cultured and plasma samples were pooled and tested by PCR. All samples were consistently positive by culture and PCR over a period of 4 days after initiation of infection. In the second model, localized infection in the thigh was induced and blood and plasma samples were taken every 3 days up to 15 days after inoculation; no samples tested positive either by culture or PCR. A similar pattern was seen in blood cultures from patients. Serum samples were collected from 14 patients who had previously had *Candida* isolated from at least one blood culture; 11 of these were PCR positive while sera from 12
patients with active oral thrush and 17 healthy volunteers were all PCR negative. This protocol was the first described that could detect and identify numerous species of Candida and was shown to work with samples from both mice and patients. More importantly the PCR could detect candidaemia without false positives generated by the presence of Candida either as a commensal or in a non-disseminated infection.

Crampin and Matthews (1993) also used a low copy number gene for PCR amplification, heat shock protein 90 (HSP90). Primers designed from the C. albicans gene generated amplicons with C. albicans DNA. In addition, non-specific products were seen in samples ‘spiked’ with C. glabrata, C. parapsilosis, C. guilliermondii, C. krusei and human DNA, although the authors stated that these were not seen in PCR performed on any clinical samples. Southern analysis allowed detection of 100 c.f.u. C. albicans ml⁻¹ broth or 5 pg genomic DNA. An investigation of 100 clinical specimens (including swabs, urines, peritoneal fluids, pus and blood/serum) tested by routine culture, extended culture and PCR, gave positive results with 23%, 31% and 37% of samples respectively. The clinical relevance of detecting C. albicans in these samples was not discussed.

One of the C. albicans chitin synthase genes (CHS1) has also been exploited as a target for PCR based on the rationale that CHS1 is not present in any mammalian genome and could therefore help to prevent false-positive results (Jordan, 1994). The single pair of primers described produced different sized amplicons from C. albicans (122 bp), C. parapsilosis (311 bp), C. tropicalis (519 bp) and C. glabrata (535 bp); these four species are responsible for 90% of cases of neonatal candidaemia. This was the first published method that was capable of detecting different medically important species of Candida in just one step. Species-specific probes were designed for use in Southern hybridization which gave a detection limit of 10 c.f.u. ml⁻¹ blood. Comparing PCR and blood culture in 27 pairs of blood samples showed concordance in 26 pairs; blood samples from 29 high-risk neonates with no signs of Candida infection and five bone marrow transplant recipients with mucosal colonization with Candida were all negative by PCR. While this protocol was not shown to be more sensitive than blood culture in this case, it had the advantages of speed and easy species differentiation.

Meanwhile, the rRNA genes remain a popular target for PCR. Holmes et al. (1994) described two sets of primers based on the 5S rRNA gene and the adjacent non-transcribed intergenic spacer (IGS); one pair amplified a product of 105 bp from C. albicans and five non-albicans species, while the second set amplified a 684 bp product from C. albicans only, with a sensitivity comparable to the PCRs already described of approximately 15 c.f.u. ml⁻¹ blood.

Similarly, the V4 region within the SSU rRNA gene was the basis for a PCR which, in conjunction with Southern blotting and a species-specific probe, could specifically detect C. albicans candidaemia in neutropenic mice
with a sensitivity of 10–15 c.f.u. ml⁻¹ blood (van Deventer et al., 1995). Gastrointestinal colonization was established in immunocompetent mice and blood samples were tested by the same method to demonstrate that the PCR would only detect invasive fungal cells and not commensals. A comparison of PCR with blood culture demonstrated a large increase in the number of positives when PCR was used (89–100% compared with 44–100% for culture). Only C. albicans was investigated in this report but the authors indicated the possibilities for adapting this method to other species using different probes.

Fujita et al. (1995) reported a universal PCR that was capable of detecting any fungal species with primers based on sequences from the 5S rRNA gene and adjacent ITS region; specific probes designed from the ITS2 region (found between 5S and 26S) were used in an enzyme immunoassay to specifically detect PCR products from C. albicans, C. tropicalis, C. parapsilosis, C. krusei and C. glabrata. Using colorimetric detection instead of ethidium bromide staining increased the sensitivity ten-fold from 10² to 10 cells ml⁻¹.

Haynes et al. (1995) and Haynes and Westerneng (1996) used a reverse primer based on a sequence from the large subunit (LSU) rRNA gene, universally conserved within the fungal kingdom, in tandem with four different forward primers, each specific for a medically important species of Candida. The four PCRs used the same temperature cycles and so could run concurrently, and were shown to correctly identify nine isolates of C. albicans, 18 of C. glabrata, 13 of C. parapsilosis and 18 of C. krusei in a blind study. The testing process, including DNA extraction from single colonies, was estimated to take approximately 3 h, although no testing was performed on clinical samples.

Recently a PCR based on the C. albicans L₁A₁ gene was described which was capable of amplifying DNA from a variety of yeasts including C. albicans, ten non-albicans Candida species, S. cerevisiae and Blastoschizomyces capitatus (Morace et al., 1997). The PCR had a sensitivity of 200 fg Candida DNA and seven different species were identified by restriction enzyme mapping. This method was applied to a small number of blood and BAL samples from patients considered likely to have fungal infection; 15 of 21 bloods and six of 20 BAL samples gave PCR-positive results and this was considered to correlate well with culture results. The authors emphasized that this protocol must be used to analyse a much larger number of patient specimens before any conclusion can be made. These results were encouraging but a much simpler extraction method would be necessary for this protocol to be used on a routine basis.

12.3.2 Invasive aspergillosis

Members of the genus Aspergillus are ubiquitous saprophytes commonly found in soil and decaying vegetation. Aspergillus conidia account for
0.1–22% of total airborne spores and are found in air and dust within the hospital environment (Anassie, 1992). There are at least 200 species of Aspergillus but few are associated with disease in humans; the majority of invasive disease is due to A. fumigatus and can arise from colonization or de novo infection (Rinaldi, 1983). The common route of entry into the body is via the respiratory tract. Conidia are smooth and, in the case of A. fumigatus, only 2–3 µm in size and can therefore easily reach the alveolar airspace; this may be directly linked to A. fumigatus being the primary pathogen of the genus (Kwon-Chung and Bennett, 1992). In predisposed (e.g. neutropenic) patients the infection rapidly becomes invasive; pulmonary disease disseminates haematogeneously through the body to other major organs and the associated morbidity and mortality are very high (Cohen, 1990). Early administration of antifungal therapy is essential for improvement of prognosis in cases of invasive disease (Burch et al., 1987), however, early diagnosis remains notoriously difficult. Routine microbiological investigation involves culture and cytological examination of respiratory specimens such as BAL fluid and induced sputum, procedures that have been evaluated and reviewed by several groups in a number of retrospective studies. Nalesnik et al. (1980) suggested that even a single Aspergillus-positive sputum culture must be treated as potentially significant in patients with haematological malignancy. Yu et al. (1986) confirmed this, adding that in neutropenic cases positive respiratory specimens were virtually diagnostic. Cytological examination of BAL specimens has been considered to be of a higher predictive value than culture (Levy et al., 1992), although in patients with acute leukaemia, BALs have been reported to be of more use for diagnosing P. carinii pneumonia than invasive aspergillosis (Saito et al., 1988). The majority of respiratory specimens are negative for Aspergillus by cytology and culture, and the predictive value of immunological diagnosis has yet to be proven. The gold standard remains cytology and culture of biopsy specimens, but open surgery is contra-indicated in critically ill patients.

In order to avoid the invasive procedures of obtaining specimens such as BAL fluid or bronchial washings, Reddy et al. (1993) investigated the possibility of using PCR to detect A. fumigatus in urine samples. The primers were based on an A. fumigatus partial protein sequence that shared a high degree of homology with ribotoxins of A. restrictus and with alpha sarcin of A. giganteus. The PCR amplified A. fumigatus and A. restrictus DNA only, with a detection limit of 0.6 pg by Southern analysis. Prospective screening of 13 urine samples from patients undergoing bone-marrow transplantation revealed two positives; however only one was from a proven case of IA.

Spreadbury et al. (1993) were able to specifically detect A. fumigatus DNA in three of three culture-positive respiratory specimens from patients with clinically diagnosed IA by using a PCR that amplified part of the 26S intergenic spacer region from the A. fumigatus rRNA gene complex. However, as Aspergillus conidia are inhaled by the general population on a day-to-day basis, the significance of finding Aspergillus in the respiratory
tract of an individual is still controversial. To assess the significance of a positive PCR result, culture-negative samples from immunosuppressed patients at high risk and from immunocompetent patients with known lung disease (other than IA) were analysed by PCR: two of ten of the former patient group and two of seven of the latter patient group gave positive results. It was suggested that results based on Southern hybridization may be too sensitive – the two positives from the former patient group were not detected by electrophoresis alone – but that the high level of correlation between culture, clinical data and PCR results supported PCR as a valid method of diagnosis of IA.

These findings were corroborated by further work from the same group; Tang et al. (1993) used primers based on sequences obtained from the alkaline protease (Alp) genes of *A. fumigatus* and *A. flavus* to detect *Aspergillus* DNA in four BAL samples from four patients with proven or probable aspergillosis. One patient with possible aspergillosis was PCR positive, as were one of 18 (6%) immunosuppressed patients with no evidence of fungal infection and five of 28 (18%) immunocompetent patients, the latter most likely resulting from colonization.

Verweij et al. (1994) investigated a larger group of low-risk patients and found a similar level of prevalence of detectable *Aspergillus* colonization. 72 BAL specimens from 70 non-neutropenic patients were tested by culture and genus-specific PCR: 11 of the 72 samples (15%) were PCR positive. In a separate study the validity of the PCR was assessed in a mouse model of IA and in patient BAL samples. The primers were derived from sequences obtained from the 18S rRNA genes of *A. fumigatus*, *A. flavus*, *A. terreus*, *A. nidulans* and *A. niger* so that other species that have been reported as opportunistic pathogens could also be detected (Cohen, 1990). Strong cross-reactions were observed with *Paecilomyces variotii*, *Penicillium marneffei* and *Penicillium chrysogenum* but these were differentiated from *Aspergillus* by Southern analysis and restriction enzyme digestion. Sensitivity was increased from 1 pg to 10 fg when rRNA was transcribed into cDNA prior to amplification, although this was deemed unnecessary as no further samples were identified as positive using this extra step. Five of six mice with experimental IA were PCR positive, as were nine of 18 BAL samples from immunosuppressed patients; none of the control mice or 14 BAL samples from low-risk patients were positive. In comparison only one BAL sample from four patients with documented IA grew *Aspergillus*.

A competitive PCR was described by Bretagne et al. (1995) which used an internal control to indicate false-negative results due to inhibition of the PCR. This was achieved by spiking each sample with a fragment of M13mp18 phage DNA flanked at each end by the *A. fumigatus*-specific primers; successful amplification would generate a 135 bp product from *A. fumigatus* DNA and a separate smaller amplicon from the control sequence. False-positive results due to carry-over contamination from previous amplifications was avoided by the substitution of dUTP for dTTP and digestion.
with uracil-N-glycosylase. However, it should be noted that this is not necessarily sufficient to prevent false positives due to contamination. In a study of PCR protocols used in seven centres for *Mycobacterium tuberculosis* detection, this substitution method was employed by the centre that had the highest rates of false-positive results (Vaneechoutte and van Eldere, 1997). Of 55 BAL samples tested with this PCR, 15 were positive, 37 were negative and three were negative due to inhibition and discounted. Three patients had proven IA and another three were high risk, but the remaining nine positive samples were from two groups of patients, none of whom developed IA. As the three patients with aspergillosis had already been clinically diagnosed and given empirical antifungal therapy, the authors concluded that PCR was of little value in diagnosing cases of IA where clinical symptoms were overt; routine methods were sufficient and the question of the predictive value of PCR diagnosis in high-risk patients remained unanswered. Verweij suggested that optimized techniques and sampling schedules are required if PCR is to prove useful in the early diagnosis of IA (Verweij et al., 1996).

Recently, Yamakami et al. (1996) reported a genus-specific nested PCR based on 18S rRNA genes of *Aspergillus* which was evaluated using serum from experimentally infected mice and from patients with IA. Southern analysis increased the sensitivity of the PCR to 5 fg *Aspergillus* DNA. Serum samples from animals sacrificed over a period of 4 days post-infection were tested by PCR and Pastorex *Aspergillus* latex agglutination; these tests gave five of eight and three of eight positives respectively and all the controls were negative. However, mice were inoculated with viable conidia through a surgical incision over the trachea, possibly allowing direct inoculation of the bloodstream, in which case this would not be an appropriate model of IA. Serum samples (100 µl) from 20 patients with documented IA were also tested, giving 14 positive samples by PCR and 12 positive samples by Pastorex. Sera from 20 healthy volunteers were all negative. These results are promising but care would need to be taken when designing sampling schedules, especially when sample volumes are so small.

A test proven to be sensitive and specific that can be performed on clinical material obtained without invasive procedures is still needed to improve the ante-mortem diagnosis of IA.

### 12.3.3 Pneumocystis carinii pneumonia

Interest in the ubiquitous and, until recently, taxonomically ambiguous *P. carinii* (Pixley et al., 1991; Stringer et al., 1992) was re-awakened in the early 1980s when the advent of AIDS caused a significant increase in the incidence of *P. carinii* pneumonia (PCP) to such an extent that PCP became a diagnostic feature of AIDS and was a major cause of morbidity and mortality in AIDS patients (Cushion et al., 1994). The major obstacle yet to be overcome in the development of simple, rapid diagnostics for this infection is the lack of an *in vitro* culture system, primarily due to the organism’s
fastidious nature (Wakefield et al., 1990). Conventional diagnostic procedures remain: cytochemical staining of BAL or, less frequently, induced sputum samples, either non-specifically (Giemsa, methenamine silver) or with a specific immunofluorescent monoclonal antibody stain (Cartwright et al., 1994). However, small numbers of organisms are difficult to visualize and a more sensitive method is needed to attempt diagnosis at an earlier stage of infection. Many research groups have therefore explored the possibilities of using PCR as a diagnostic method for PCP.

The first PCR reported for detection of *P. carinii* in clinical samples amplified a sequence from the large subunit mitochondrial rRNA gene (mt rDNA) and although sequences were derived from a rat isolate, the PCR also amplified *P. carinii* DNA from human isolates (Wakefield et al., 1990). The products were verified using Southern analysis with a probe specific for human *P. carinii*. The PCR appeared to be at least as sensitive as staining when detecting *P. carinii* in BAL, but apparently false-positive signals were observed on blots from three immunosuppressed patients with no evidence of PCP and from bronchoscope washings from an unspecified source.

While some groups have designed novel PCRs to detect *P. carinii*, the mt rDNA primers have since been used by many other investigators. Eisen et al. (1994) found this PCR to be more sensitive than Toluidine Blue-O staining and immunofluorescence in sputum samples from 20 HIV-positive patients. In another study an enzyme immunoassay was developed as a potential replacement for Southern analysis (Cartwright et al., 1994). This assay detected *P. carinii* in all induced sputa from documented PCP cases, compared with 78% by immunofluorescence, and the entire procedure could be performed in a single day. This PCR assay was considered sensitive enough to detect *P. carinii* in induced sputa from patients with a low organism burden, possibly saving them from the invasive procedure of bronchoscopy. However, no advantage over conventional methods for routine detection of *P. carinii* in BAL was evident. These findings are similar to those reported by other groups (Lipschik et al., 1992; Tamburrini et al., 1993; Roux et al., 1994).

Honda et al. (1994) described a comparison of conventional and capillary PCR in which both protocols used the mt rDNA primers to detect *P. carinii* in sputum and BAL samples from immunosuppressed patients. The volume of the reaction mix in capillary PCR is only 10 µl (compared with 25–100 µl in conventional PCR) and thermocycling is performed in sealed glass capillary tubes; this allows much faster and more efficient temperature transfer. However, savings made in reaction constituents have to be balanced against the purchase of a specialized thermocycling machine. The capillary PCR was 1000-fold more sensitive than conventional PCR (detecting $3 \times 10^{-2}$ fg DNA compared with 30 fg) and took only 20 min to complete. The authors suggested that the high sensitivity of the capillary protocol enabled detection of *P. carinii* carriage and subclinical infection and allowed for earlier initiation of antimicrobial therapy.
In an attempt to monitor organism loading during treatment of \textit{P. carinii} infection in immunosuppressed rats, O’Leary \textit{et al.} (1995) incorporated the mt rDNA primers into a semi-quantitative PCR (SQPCR). Rat β-globin was used as the internal standard and assay results were expressed as \textit{Pneumocystis} mt rDNA/globin signal ratios. The SQPCR results correlated strongly with cyst counts in BAL and lung homogenates. Furthermore the PCR was capable of detecting \textit{P. carinii} DNA during the early phase of infection when few cysts could be visualized by staining of pulmonary tissue and none were seen in BAL fluid.

Alternative gene targets for \textit{P. carinii} PCR include those encoding the 5S rRNA (Kitada \textit{et al.}, 1991), 18S rRNA (Lipschik \textit{et al.}, 1992), dihydrofolate reductase (DHFR) (Schluger \textit{et al.}, 1992) and thymidylate synthase (TS) (Olsom \textit{et al.}, 1993) genes. These were all included in a comparison of the efficacy of different PCR methods, along with the mt rDNA PCR and a nested PCR which amplified the ITS of the rRNA genes (Lu \textit{et al.}, 1995). When used to analyse 50 BAL specimens, the two nested PCRs (ITS and 18S) achieved 100% sensitivity after the second round (53% and 50% respectively after the first), the mt rDNA PCR 87%, the 5S rDNA PCR 33%, the TS PCR 60% and the DHFR PCR 23%. The 18S and TS PCRs demonstrated false-positive results with \textit{S. cerevisiae} and \textit{C. albicans}, plus \textit{Cr. neoformans} and \textit{C. albicans} respectively, leading to the conclusion that the nested ITS PCR was the most sensitive and specific of the methods tested.

In order to avoid the invasive nature of sampling methods such as bronchoscopy or open lung biopsy, non-invasive samples such as serum and blood have been investigated as attractive alternatives for potential routine specimens when diagnosing \textit{P. carinii} infection. Preliminary experiments by Kitada \textit{et al.} (1991) detected \textit{P. carinii} DNA in three of five mice with experimentally induced \textit{P. carinii} infection; reduced sensitivity compared with performance of PCR with lung biopsy specimens was attributed to low organism load in the blood. Schluger \textit{et al.} (1991) used the DHFR PCR on serum samples from AIDS patients with PCP; \textit{P. carinii} DNA was detected in five of 14 experimental rats and seven of 18 AIDS patients. However, four patients had documented extrapulmonary PCP, two of which were not detected by PCR. Tamburrini \textit{et al.} (1993) successfully detected \textit{P. carinii} in spiked samples down to two organisms µl⁻¹ serum or whole blood, but this could not be reproduced in serum and blood specimens from HIV-positive patients with documented PCP. The conclusion was that, even allowing for haematogeneous dissemination, the presence of \textit{P. carinii} in the bloodstream is transient and PCR detection is not applicable to serum samples.

### 12.3.4 Cryptococcosis

Cryptococcal meningitis, caused by \textit{Cr. neoformans} var. \textit{neoformans} or \textit{Cr. neoformans} var. \textit{gattii}, is a major cause of morbidity in individuals infected with HIV (Kwon-Chung \textit{et al.}, 1994). Before the cryptococcal antigen latex
agglutination (LA) test became widely available, cryptococcal meningitis was diagnosed by India ink preparations of cerebrospinal fluid; this gave a positive result in approximately 50% of cases. Detection of capsular polysaccharide by LA has a reported sensitivity and specificity of 99% and immunological diagnosis appears to be reliable (De Repentigny et al., 1994).

The reliability of the LA test as a method of diagnosing cryptococcosis has obviated the need for diagnostic PCR. However, a number of protocols have been described. Mitchell et al. (1994a) designed primers from the 5.8S rDNA and adjacent ITS region of Filobasidiella neoformans, the teleomorph of Cr. neoformans. These primers were able specifically to amplify DNA from 37 strains of Cr. neoformans; amplification from clinical material was suggested but not attempted. A nested PCR based on the Cr. neoformans URA5 gene was developed to diagnose non-HIV related pulmonary cryptococcosis (Tanaka et al., 1996). Sixteen respiratory specimens were analysed in order to evaluate both the PCR and the glass bead-based extraction method. Of the five which were positive by culture, four were PCR positive and no culture-negatives were PCR positive. While this protocol performed well on this small number of samples, the advantages over culture were unspecified.

12.3.5 Dimorphic fungal infections

The dimorphic fungi H. capsulatum, B. dermatitidis, Co. immitis and Pa. brasiliensis are primary human pathogens. However, the incidence of each has increased as the number of immunosuppressed patients has risen. The route of infection for these is generally via the respiratory tract; they cause primary pulmonary disease in competent hosts. H. capsulatum causes chronic pulmonary disease in those with structural lung defects and disseminated disease in immunocompromised hosts. Co. immitis is asymptomatic in 60% of cases and rarely disseminates. Pa. brasiliensis manifests more as a disseminated infection than a pulmonary one while B. dermatitidis infection is initially pulmonary but can disseminate and mimic other diseases such as tuberculosis (TB) (Bradsher, 1996).

While the incidence of these fungi is increasing, particularly in the Americas where they are endemic, diagnosis using molecular methods is not well documented and little diagnostic PCR research has been published. Diagnosis relies mostly on cytological staining of BAL fluids and sputa with Papanicolaou stain and methenamine silver; TB is excluded by the acid Schiff test (Lemos et al., 1995). Visualization of the fungus gives a firm diagnosis for histoplasmosis and blastomycosis as colonization with these organisms does not occur (Bradsher, 1996).

Molecular methods other than PCR have been successfully used; a chemiluminescent DNA probe kit (GenProbe, San Diego, California) has been shown to have sensitivities of 87.8–100% and specificities of 100% when identifying different isolates of B. dermatitidis, H. capsulatum, Co.
immittis and Cr. neoformans (Stockman et al., 1993). The probes, based on rDNA sequences, were found to shorten the diagnostic process significantly, and the preparation required was much less than for exoantigen testing. These probes were recommended by the author to supplement diagnosis by in vitro conversion of hyaline mould to yeast forms; false-negative results that occasionally occurred with the B. dermatitidis probe could thus be avoided and morphologically similar organisms could be differentiated, such as the yeast form of H. capsulatum and C. glabrata.

12.4 Future Prospects for PCR Diagnostics in Medical Mycology

Commercially produced PCR-based protocols are now widely available for the diagnosis of various infectious diseases, e.g. TB, viral hepatitis. Notwithstanding the huge research investment that has been made in the development of diagnostic PCRs in the field of medical mycology, there are no such protocols available for the diagnosis of fungal infections. The most likely contender for a successful PCR-based protocol is the detection of P. carinii for the diagnosis of PCP, but the advantages of PCR over established diagnostic methods for this disease are debatable. Until simple, rapid and reproducible methods are developed for the extraction of fungal DNA from clinical material, the routine diagnosis of systemic fungal infections by PCR remains unlikely.

References


Applications of PCR in Fungal–Plant Interactions

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13.1 Introduction

This chapter describes the use of PCR-based techniques for studying plant–fungal interactions. The first section describes the uses of PCR for detection of fungal pathogens and symbionts within their hosts and the other three sections cover approaches used to study the genes involved in plant–fungal interactions. Some genes involved in fungal infection and plant responses to infection are common to many host–pathogen combinations and some of these have been characterized at the nucleotide or amino acid level. Where this is the case, PCR allows similar genes from other interactions to be isolated and characterized relatively easily. A second approach, which does not rely on any previous sequence characterization, is to search for genes whose expression is altered during fungal interactions and PCR-based methods are available to do this. The final application discussed is the use of PCR to detect genomic differences between plants or fungi that differ in one characteristic only, e.g. fungal pathogenicity or plant resistance to a particular pathogen. Recent reviews on various aspects of plant–fungal interactions have described: plant resistance genes (Ellis et al., 1988; Michelmore et al., 1992; Keen, 1992); fungal pathogenicity and virulence genes (Schäfer, 1994; Van Etten et al., 1994; Hensel and Holden, 1996); fungal avirulence genes (de Wit, 1992); genes involved in mycorrhizal interactions (Gianinazzi-Pearson, 1996; Gianinazzi-Pearson et al., 1996); plant defence genes (Dixon et al., 1994); and fungal colonization of plants (Takenaka, 1995).
13.2 Applications

13.2.1 Detection, localization and quantification of fungi in plant tissues

PCR is now widely used to detect and identify microorganisms, and there are many examples of its use for fungi, including both plant pathogens and symbionts. This application has been reviewed by Foster et al. (1993), Ward (1994) and Bridge and Arora (Chapter 4) and will not be covered here. PCR assays developed for a fungus in pure culture can usually be adapted for detection of the fungus within the plant although procedures to overcome PCR inhibitors in some plant tissues may be necessary (Robb and Nazar, 1996). This then opens the way for using PCR to quantify the extent of fungal colonization of plant tissue or to localize the fungus within the plant.

One area in which PCR is beginning to be used is the quantification of fungal biomass in infected tissues. For reviews of quantitative PCR, see Ferre (1992), Diaco (1995) and Zimmerman and Mannhalter (1996). The use of PCR for quantification is not straightforward. This is because the PCR includes an exponential phase, so minute differences in efficiency of reaction between samples can give rise to dramatically different amounts of final product. To overcome this problem, internal control DNA, which is amplified in the same PCR reaction, should be included. The quantity of control product is then used to adjust the quantity of the test amplicon for variations in the efficiency of the PCR between tubes. The detection and quantification of the PCR products can be done by measuring the intensity of ethidium bromide-stained DNA on gels (e.g. using densitometry), by scintillation counting of labelled DNA, or by using colorimetric techniques.

Quantitative PCR has several advantages over other methods of determining fungal biomass, such as tissue maceration and dilution plating which is a lengthy and labour-intensive procedure. Also, the dilution plating assay may only indicate the amounts of fungus that can be isolated rather than the amount actually present, and it cannot be used for obligate pathogens or symbionts. PCR is a much more rapid technique and it does not require isolation of pure fungus from the tissue. Another method of biomass determination is to measure ergosterol or chitin levels, but this method does not allow discrimination between different fungi infecting the same plant, which is possible with PCR. Serological methods have also been used for fungal biomass determination but it is generally difficult to obtain antibodies with the required specificity for the fungus being studied. PCR has the potential to measure fungal biomass simply and with great sensitivity and specificity. Quantitative PCR has been used in the detection of mycorrhizal fungi (Simon et al., 1992), Verticillium spp. (Hu et al., 1993; Moukhamedov et al., 1994; Robb and Nazar, 1996), Leptosphaeria maculans (Mahuku et al., 1995) and Microdochium nivale (Nicholson et al., 1996). The ability to monitor the amounts of different fungi infecting the same plant at the same time by quantitative PCR will be of great value in studying disease complexes.
(Nicholson et al., 1996) and interactions between fungi, e.g. synergism and competition.

Localizing fungi within plant tissues can be done simply by separating the different parts of the plant and testing each for the presence of the fungus; PCR was used to monitor the spread of *Verticillium* species through their hosts at different times after infection (Moukhamedov et al., 1994; Hu et al., 1993). A much more precise method, capable of localizing the fungus at the cellular level, is *in situ* PCR (Nuovo, 1995), in which thin sections of infected plant tissue are placed on a microscope slide and the PCR is performed directly on this. This is technically difficult and although there are now many applications in studying human infections, particularly those involving viruses, we are not aware of any applications to the study of fungi or plant–fungal interactions published yet. This could be an area for development in the future, not just for detection of the fungi themselves but also for the detection of mRNA from genes expressed in plant–fungal interactions.

### 13.2.2 Targeted approaches to cloning and analysing plant and fungal genes

These approaches can be used when physiological, biochemical and/or molecular data about a gene or gene product suggest that it is involved in a particular plant–fungal interaction. They are particularly suitable for genes and gene products that are common to many plants or fungi and/or are expressed in response to a range of pathogens, hosts or other stimuli. Many of the plant defence genes fall into this category. Nucleotide or amino acid sequence data from previously characterized genes or proteins are used to design primers that can amplify the same or homologous genes or identify cDNA clones for those genes. Whether designing primers from amino acid or nucleotide sequences, this approach usually involves the use of degenerate primers that allow for uncertainties in the nucleotide sequence (see Kwok et al., 1995 for a review of their design and use). Examples of genes isolated using targeted approaches are given in Table 13.1.

Where a protein involved in a particular interaction has been characterized, amino acid sequence information can be used to design primers to amplify part of the corresponding gene from genomic DNA or cDNA. Because of the redundancy of the genetic code, a given amino acid may be encoded by different nucleotide triplets. Degenerate primers should be designed as a pool of all the possible combinations of nucleotides that could code for the amino acid sequence. Deoxyinosine can be used to reduce the complexity of the primer pool. In other cases, nucleotide sequence information can be used to design primers, e.g. where nucleotide sequence information is already known for related genes. This generally involves the use of degenerate primers because even within conserved regions of genes there is often some variation, e.g. between different plants or fungi or between different members of a gene family in the same plant.
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<th>Genes isolated from various plant–fungal interactions using PCR-based targeted approaches.</th>
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<td>Fungal and plant genes involved in mycorrhizal interactions</td>
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Once a small region of the gene of interest has been amplified using PCR, full-length clones may be obtained by screening genomic or cDNA libraries with labelled PCR-amplified DNA, or by using other PCR-based approaches. For cDNA clones the missing parts of the gene sequence can be cloned by an approach most commonly referred to as RACE (rapid amplification of cDNA ends; Frohman, 1995). RACE is also known as anchored PCR or one-sided PCR. RACE involves amplification using one primer derived from a short stretch of known sequence in the gene/mRNA and another primer that anneals either to the poly(A)-tail (to obtain the 3′-end) or to an appended homopolymer tail (for the 5′-end). There are several examples of its use in cloning genes involved in plant–fungal interactions (Vad et al., 1993; Nielsen et al., 1994, 1996; Murphy and Walton, 1996).

PCR-based approaches have allowed genes and their mRNA products to be cloned and analysed much more quickly than was previously possible. Once sequences are published it is possible for other workers to use the information to analyse similar genes immediately rather than waiting for the exchange of clones. There are various uses for the genes once isolated, in addition to characterizing the gene structure and organization. The spatial and temporal expression of the gene can be studied during infection by techniques such as Northern blotting (Vad et al., 1993; Wu et al., 1994; Zhu et al., 1995; Nielsen et al., 1996), reverse transcriptase (RT) PCR (Båga et al., 1995; Guo et al., 1995; Meyer et al., 1996) or in situ hybridization. The gene can be cloned into an expression vector allowing purification and further characterization of the protein and the production of antisera (Templeton et al., 1994; Guo et al., 1995). The promoter from the gene can be fused to a reporter such as β-glucuronidase (GUS), transformed into plants and the expression studied in various tissues at different times after infection or other treatments (Zhu et al., 1995).

In fungi, mutants lacking particular genes have been obtained by targeted gene disruption using cloned genes and this has allowed functional studies to be carried out. A gene encoding the exo-β-1,3-glucanase of Cochliobolus carbonum was cloned using degenerate primers based on amino acid sequence and this was then used to generate mutants that could not produce the enzyme. The mutant was still pathogenic to maize, indicating that the exo-β-1,3-glucanase gene was not essential for pathogenicity (Schaeffer et al., 1994). A similar approach was used to study an extracellular protease ALP1 from Cochliobolus carbonum (Murphy and Walton, 1996). The ALP1 mutants thus generated had the same growth and disease phenotypes as the wild-type strain, indicating that this gene by itself was not required for pathogenicity. Although the above studies could have been done without the PCR, its use enabled the work to be done much more easily and quickly than was previously possible.

PCR can also be used for in vitro mutagenesis to study the effects of particular amino acid residues on the biological activity of the gene product. Altered primers can be used to replace the nucleotides coding for single or
multiple amino acid residues in the protein. This approach was used to study the mode of action of the AVR9 elicitor peptide involved in the *Cladosporium fulvum* – tomato interaction (Honée et al., 1994).

### 13.2.3 Studying plant and fungal genes involved in interactions by comparative genome analysis

The biochemical basis of the effects of most genes involved in plant–fungal interactions is not known. One approach devised for studying these genes is based on comparing the DNA of organisms with or without a trait of interest to find the regions that differ. In particular, this has been used to study plant resistance genes, but the methods described are applicable to other plant and fungal genes. The first stage in the analysis is to find a molecular marker that is closely linked genetically to the required gene. Two types of strategies are commonly used to ‘home in’ on the region of interest. The first is to use near isogenic lines, that differ primarily in the gene of interest (Horvath et al., 1995). The second is a ‘pooling approach’ such as bulked segregant analysis (Michelmore et al., 1991). Using this technique, markers linked to a trait of interest are identified using two pooled DNA samples, one from (homozygous) individuals that express the trait and the other from (homozygous) individuals lacking the trait. Any polymorphism between the two pools should be linked with the trait. Markers thus identified are then confirmed by mapping a segregating population.

Until the advent of PCR in the late 1980s most of the markers used were RFLP (restriction fragment length polymorphisms). Although these allowed much faster and easier screening than did the previously available phenotypic markers, the procedure required relatively large quantities of purified DNA and usually radioactive detection. PCR offered the opportunity of being able to screen for differences much more easily; the techniques do not involve radioactivity, are simpler and can be performed on small amounts of crudely prepared samples. Some of the previously developed RFLP methods were subsequently converted to PCR-based protocols by sequencing the clones and then designing primers based on these sequences (Balint-Kurti et al., 1994; Kilian et al., 1994). These markers are frequently referred to as SCARs (sequence-characterized amplified regions).

Additional PCR-based methods were also developed that allow the identification of useful markers much more quickly. The most widely used of these involves PCR at low stringency, with arbitrarily chosen primers, and is known by the acronyms RAPDs (random amplified polymorphic DNAs; Williams et al., 1990), AP-PCR (arbitrarily primed PCR; Welsh and McClelland, 1990) and DAF (DNA amplification fingerprinting). The concept is essentially the same and hereafter RAPDs will be used to refer to all three techniques. In this technique, the primers are usually much shorter (10 bases) than those generally used for PCR (~20 bases) and one primer is...
sufficient. Also the conditions of the PCR are made relatively non-specific, e.g. by reducing the annealing temperature and increasing the number of amplification cycles (e.g. from 25 to 45). Since the primers are so short and mismatches are allowed by low stringency conditions it is likely that many primer-binding sites will be present in the target DNA. There is also a high probability that pairs of sequences complementary to the primer will be arranged close enough together and in the correct orientation for PCR-amplification of the intervening sequences. Each RAPD is likely to result in the amplification of several (usually 3–10) bands that can be detected by agarose gel electrophoresis and ethidium bromide staining. Alternative detection methods, that reveal a larger number of bands, are silver staining of polyacrylamide gels (Caetano-Anollés et al., 1991) radioactive labelling of products run on acrylamide gels detected by autoradiography (Welsh and McClelland, 1990) and denaturing gradient gel electrophoresis (DGGE) (Procunier et al., 1995). Reactions with different RAPD primers can detect variation at different levels, including species, isolates and near-isogenic lines differing only in a single trait, e.g. plant resistance to disease or fungal pathogenicity.

Due to the ease of use and the speed at which markers can be developed using it, RAPDs have been used extensively in the few years since the method was first reported. Several reviews have covered various aspects of the technique and its applications in genetics and plant breeding (Waugh and Powell, 1992; Bowditch et al., 1993; Rafalski and Tingey, 1993; Williams et al., 1993; He et al., 1994; McClelland and Welsh, 1995). Using RAPDs, markers have now been identified that are closely linked to the resistance genes for barley stem rust (Horvath et al., 1995), barley powdery mildew (Xu and Kasha, 1992), wheat leaf rust (Schachermayr et al., 1994), lettuce downy mildew (Michelmore et al., 1991; Paran and Michelmore, 1993; Maisonneuve et al., 1994), pea powdery mildew (Timmerman et al., 1994), bean rust (Miklas et al., 1993) and muskmelon Fusarium wilt (Wechter et al., 1995). RAPDs have also been used as markers in fungi, but the work here has mainly been used for species and population analysis (McDermott et al., 1994).

The major problem with the RAPD technique is the lack of reproducibility, although with appropriate precautions the effects of this can be minimized. Usually RAPD bands behave as dominant markers (only one of the alleles can be amplified) rather than co-dominant markers (each allele amplifies a different band) which would be generally more useful. Another feature of RAPDs is that multiple loci are amplified. Whilst this is useful initially, in that many loci can be analysed at once, it is a disadvantage in subsequent analyses because of the complexity of the patterns. Another potential source of problems is contamination and it is essential to include negative controls. Because of these problems RAPD markers are often converted to more reproducible markers (SCARs) after cloning and sequencing (Markussen et al., 1995; Naqvi and Chattoo, 1996).
Another, more recently developed technique for isolating markers linked to traits of interest is AFLPs (amplified fragment length polymorphisms) (Vos et al., 1995; Perkin Elmer, 1995). The technique is based on the selective amplification of restriction fragments generated from the genomic DNA. First the DNA is digested with restriction enzymes and oligonucleotide adaptors are ligated to it. Sets of restriction fragments are then amplified selectively and, finally, the amplified fragments are analysed after gel electrophoresis. The selectivity is achieved by using primers that contain the restriction site and variable 3’ extensions. The number of bands produced depends on the type of gel analysis used but typically 50–100 restriction fragments are resolved on denaturing polyacrylamide gels. The AFLP method is more robust and reliable than RAPDs because more stringent reaction conditions are used, but is more difficult and expensive to do. The technique has been used to select markers closely linked to the $R1$ (resistance to Phytophthora infestans) gene of potato (Meksem et al., 1995) and the tomato Cf-9 gene (resistance to Cladosporium fulvum; Thomas et al., 1995).

Other PCR-based methods have also been developed that allow rapid identification of markers for genes of interest (for reviews, see Karp et al., 1996; Staub et al., 1996), including variable non-translated repeats (VNTRs) and microsatellite/simple sequence repeats (SSRs). Once developed, these markers are useful in screening breeding lines to track which progeny contains the desirable trait (marker-assisted selection). Molecular markers, and particularly those involving PCR, allow much faster and easier screening than do the previously available phenotypic markers, and this is important in these high throughput studies.

The markers can also be used to facilitate cloning of the gene of interest (for resistance, pathogenicity, etc.) by map-based cloning procedures (Young, 1990, 1995; Michelmore et al., 1991). This is an approach whereby information about the gene’s chromosomal location is used as the basis for cloning. After markers that are closely linked genetically to the gene of interest have been identified a physical map is constructed in which the distances between markers are calculated as numbers of nucleotides rather than recombination frequencies (e.g. using pulsed-field gel electrophoresis). Chromosome walking is then used to identify overlapping clones in the region of interest. The last stage is to pinpoint the target gene among the overlapping clones, usually by complementation of organisms with the recessive phenotype. This approach is a long-term, labour-intensive undertaking, but it has been used to isolate the genes conferring resistance to the bacterial pathogen Pseudomonas syringae in tomato (Martin et al., 1993) and Arabidopsis thaliana (Mindrinos et al., 1994).

It may also be possible to identify genes of interest which, for example, differ between wild-type and deletion mutants, by genomic subtraction methods. DNA that is found in one of the pair of organisms under test, but not the other, is selected and then amplified using PCR (Straus and Ausubel, 1990; Lisitsyn et al., 1993). This approach has been used to clone the
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Arabidopsis gibberellin synthesis (GA1) locus (Sun et al., 1992). Subtractive hybridization is also used on mRNA, to find mRNA species which are present in colonized plants but absent from the uninfected plant (see section 13.2.4).

13.2.4 Non-targeted approaches to characterizing altered gene expression during plant–fungal interactions

The use of PCR in combination with standard cloning techniques is now a widely adopted strategy to identify genes whose expression is altered during biological interactions and it has resulted in the saving of much time, effort and cost. Several methods have been developed and optimized in different laboratories to suit a specific need and a particular biological system. In this section we discuss three applications of PCR to investigate changes in gene expression during fungal–plant interaction, without prior knowledge of the genes or proteins that might be involved: (i) the use of PCR in combination with differential hybridization methods to screen for differentially expressed genes; (ii) RNA fingerprinting methods to analyse differential gene expression; (iii) the use of PCR in conjunction with subtractive hybridization to generate probes specific to differentially expressed genes.

Differential screening of cDNA libraries

Various papers discuss the use of PCR to construct cDNA libraries (Gurr and McPherson, 1992; Clackson et al., 1993; Lambert and Williamson, 1993). PCR can also be used during screening to estimate the average cDNA insert size (Güssow and Clackson, 1989). This allows the presence, size and orientation of inserts to be determined rapidly by amplification with flanking primers, and (for orientation) by including a single internal primer. PCR can be performed directly on single phage plaques, aliquots from phage libraries, or bacterial colonies, circumventing all DNA preparation. When the PCR-based assessment is used in combination with the IPTG and X-Gal colour selection method (Sambrook et al., 1989) to determine the relative numbers of recombinant (white) versus wild-type (blue) phage, it provides a simple and accurate assessment of the quality of cDNA library under investigation.

Differential screening allows the identification of cDNA clones corresponding to differentially expressed genes. Poly(A)+ RNAs extracted from the two samples under comparison are used as templates to generate labelled cDNA probes that are hybridized separately to duplicate copies of the same cDNA library. Clones that hybridize to both probes correspond to genes that are constitutively expressed in both samples, whereas clones that hybridize to only one probe correspond to mRNAs that are expressed in only one sample (Sambrook et al., 1989). To ensure the purification of single clones, putative positive phage clones are screened through two or three rounds of differential hybridization. Due to the high number of phages
which are analysed during each round of screening, the method can be very time-consuming and costly. PCR can be used to simplify and speed up this process. An aliquot is taken from each phage clone as template for a PCR reaction using, for example, M13 forward and reverse sequencing primers to amplify the cDNA insert within the polylinker or any specific primers flanking the cloning site. There are several advantages of using this approach: (i) it shows whether the phage clone is single or contaminated by other surrounding phages; (ii) it gives an estimate of the size of the inserted cDNA; (iii) several phage clones can be screened at once; (iv) when combined with Southern blot analysis it allows analysis of the induction or repression of the clones under investigation. This approach has been used to clone symbiosis-related cDNAs from eucalypt ectomycorrhizae (Tagu et al., 1993) and to study genes whose expression is altered during the colonization of tomato roots with the arbuscular mycorrhizal fungus *Glomus mosseae* (Tahiri-Alaoui and Antoniw, 1996).

**RNA fingerprinting methods**

Another approach to detecting and isolating differentially expressed genes is by comparing cDNA fingerprints generated from mRNA expressed under different conditions (see Chapter 3; McClelland and Welsh, 1995; McClelland et al., 1995, for recent reviews and protocols). These methods are the RNA equivalents of the DNA fingerprinting methods described in Section 13.2.3. Liang and Pardee (1992) were the first to describe an RNA fingerprinting protocol, which they termed differential display reverse transcriptase PCR (DDRT–PCR). They used a primer for reverse transcription based on oligo-dT but with an anchor of two bases at the 3′-end. After reverse transcription and denaturation, PCR was performed on the first strand cDNA using the anchored oligo-dT primer together with an arbitrarily chosen 10-mer primer to generate a fingerprint of products analysed on a polyacrylamide sequencing gel. DDRT–PCT has been used in the investigation of gene regulation in both animal and plant systems. Although the principle of the technique is simple and elegant, there are several problems in practice, including the redundancy and under-representation of certain mRNA species and a rather high number of false positives that cannot be confirmed by Northern blot analysis. We have encountered such problems in our laboratory in investigating the interaction of barley roots with the take-all fungus, *Gaeumannomyces graminis* var. *tritici*, and potato infected with potato cyst nematode, *Globodera rostochiensis*. Despite extensive analysis, and although barley and potato showed dramatic physiological responses to the pathogen attack, no differences in gene expression were found between uninfected plants and plants responding to pathogen attack. This led to the design of an experimental system in which it was demonstrated that differential display showed a strong bias towards high copy number mRNA species (Bertioli et al., 1995). There are few reports of the successful use of differential display in analysing...
plant–microbe interactions (Martin-Laurent et al., 1995; Martin-Laurent et al., 1996). Various efforts have been made to optimize and streamline the method (Liang, et al., 1993, 1994; Li et al., 1994; Mou et al., 1994).

In an alternative protocol, termed RNA arbitrarily primed PCR (RAP-PCR; Welsh et al., 1992) arbitrary primers are used both for cDNA synthesis and PCR amplification. A method of RNA fingerprinting based on AFLPs has also been developed and used to analyse gene expression during potato tuber development (Bachem et al., 1996).

**Subtractive hybridization methods**

This approach involves the subtraction (by hybridization) of the mRNAs expressed in uninfected cells from those expressed in infected cells to leave only those expressed during infection. PCR has been invaluable in amplifying the minute amounts of material that result from this process. The cDNA thus produced can be used to make subtractive cDNA libraries or used as a probe to screen existing cDNA libraries. There are several variations of the technique depending on the method used to separate the hybridized and non-hybridized molecules, including hydroxylapatite chromatography (Sambrook et al., 1989), biotinylation (Wieland et al., 1990) and magnetic beads (Sharma et al., 1993). Examples of the use of the technique in studying plant–fungal interactions have been reported (Sharma et al., 1993; Lönnberg et al., 1995; Roberts and Pryor, 1995; Justesen et al., 1996). Recent developments in subtractive hybridization techniques include representational difference analysis (RDA) and PCR-select cDNA subtraction.

RDA is a method originally developed by Lisitsyn et al., (1993) to isolate differences between two complex genomes. Genomic RDA relies on the generation, by restriction enzyme digestion and PCR amplification, of simplified versions of the genomes under investigation known as ‘representations’. If an amplifiable restriction fragment (the target) exists in one representation (the tester), and is absent from another (the driver), a kinetic enrichment of the target can be achieved by subtractive hybridization of the tester in the presence of excess driver. Sequences with homologues in the driver are rendered unamplifiable, while the target hybridizes only to itself, and retains the ability to be amplified by PCR. Successive iterations of the subtraction/PCR process produces bands on ethidium bromide-stained agarose gels corresponding to enriched target. The RDA method was adapted for cDNA and successfully used to identify a number of caffeine-induced cDNA fragments from pre-B cell lines (Hubank and Schatz, 1994). Although we are not aware of any published work using the cDNA-RDA method to investigate altered gene expression in plant–fungal interactions, the approach seems to be fast, sensitive, reproducible, with few false positives and capable of being applied to a wide range of biological problems.

PCR-select cDNA subtraction (Clontech, 1996) uses a new method called suppression PCR (Siebert et al., 1995). The PCR-select cDNA primer
adaptors are engineered to prevent undesirable amplification, i.e. amplification of common sequences, during PCR. Suppression occurs when complementary sequences are present on each end of a single-strand cDNA. During each primer annealing step, the hybridization kinetics strongly favour the formation of a pan-handle secondary structure that prevents primer annealing. When occasionally a primer anneals and is extended, the newly synthesized strand will also have the inverted terminal repeats and form another pan-handle structure. Thus, during PCR, non-specific amplification is efficiently suppressed, and specific amplification of cDNA molecules with different adaptors at both ends can proceed normally. We have used this technique to generate two specific probes of mRNA that are specifically expressed in arbuscular mycorrhizal colonized tomato roots (tester) but not in control uninoculated roots (driver). The two probes were used to screen a cDNA library prepared from mRNA from arbuscular mycorrhizal-colonized tomato roots (Tahiri-Alaoui and Antoniw, 1996). Two different cDNA clones were isolated, which after partial sequencing showed 94\% similarity to an inorganic phosphate transporter from potato and 86\% similarity to the ATP phosphoribosyl transferase from *Escherichia coli* and *Salmonella typhimurium* (A. Tahiri-Alaoui and J.F. Antoniw, unpublished work).

### 13.3 Conclusions

PCR techniques have made a large impact on research into plant–fungal interactions, as they have done in many other areas of biological research. PCR-based methods have been developed which allow the detection of many plant pathogens and symbionts on or in their hosts more rapidly and reliably than before and for a few fungi these methods are quantitative. PCR has also simplified the isolation of genes involved in plant–fungal interactions. Once a protein or nucleotide sequence is available for one gene, primers can be designed to allow related genes to be isolated very quickly. However, PCR can also be used to isolate genes involved in plant–fungal interactions without prior knowledge of the proteins involved. These methods detect differences at the RNA level that correlate with fungal infection or differences at the DNA level that correlate with a trait such as plant resistance or fungal pathogenicity.

### Acknowledgements

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PCR as a Tool for the Investigation of Seed-borne Disease

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14.1 Introduction to Seed-borne Disease

Seed-borne diseases are defined as diseases which have one or more stages of their life cycle associated with the seed. For example, nearly all the major fungal pathogens of rice have been reported to be seed-borne. The range of organisms infecting seed is extensive (Tables 14.1–14.3). However, very little is known about the role and importance of seed-borne fungal inoculum in the dissemination and development of even the most common diseases. Ninety per cent of all food crops are propagated by seed, and the nine most widely grown crops in world agriculture (wheat, sugar beet, rice, maize, barley, groundnut, soybean, common (Phaseolus) bean and sorghum), are all affected by seed-borne diseases.

The importance of seed as a vector of a range of diseases is well established (Neergaard, 1977), and there is a significant economic impact of seed-borne disease, particularly in under-developed countries where routine chemical treatment of seed is prohibitively expensive, and individual farmers can suffer huge yield reductions. For example, an average 4% overall crop loss of the wheat harvest in the US could include individual farmers’ losses of over 80% in an infected field. Similarly, an average 2% overall crop loss of the rice harvest in the Philippines could include individual farmers losses of over 58% in an infected field.

Seed diseases and seed-borne diseases can be caused by a number of different factors: microorganisms (including viruses, bacteria, fungi and nematodes), physiological factors (including nutrient deficiency, growth in an unsuitable environment, phytotoxicity, ageing and congenital disorders) and mechanical factors (due to processing or handling equipment and insect injury).
### Table 14.1. Relative frequency of different organisms as seed-borne pathogens (Richardson, 1996).

<table>
<thead>
<tr>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungi</td>
<td>915</td>
</tr>
<tr>
<td>Bacteria</td>
<td>111</td>
</tr>
<tr>
<td>Viruses and viroids</td>
<td>238</td>
</tr>
<tr>
<td>Nematodes</td>
<td>13</td>
</tr>
</tbody>
</table>

### Table 14.2. Major seed-borne organisms prevalent in Nepal (Rajbhandary and Shrestha, 1992).

| Host | Causal agent            | Disease        | Loss (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice</td>
<td><em>Alternaria padwickii</em></td>
<td>Pink kernel</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Bipolaris oryzae</em></td>
<td>Brown spot</td>
<td>5—20</td>
</tr>
<tr>
<td></td>
<td><em>Pyricularia oryzae</em></td>
<td>Blast</td>
<td>10—30</td>
</tr>
<tr>
<td></td>
<td><em>Xanthomonas campestris pv. oryzae</em></td>
<td>Bacterial blight</td>
<td>10—25</td>
</tr>
<tr>
<td>Wheat</td>
<td><em>Alternaria alternata</em></td>
<td>Black point</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Bipolaris sorokiniana</em></td>
<td>Brown spot</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Tilletia caries</em></td>
<td>Bunt</td>
<td>&lt;5</td>
</tr>
<tr>
<td></td>
<td><em>T. laevis</em></td>
<td>Bunt</td>
<td>&lt;5</td>
</tr>
<tr>
<td></td>
<td><em>T. indica</em></td>
<td>Karnal bunt</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Ustilago tritici</em></td>
<td>Loose smut</td>
<td>5—10</td>
</tr>
<tr>
<td>Maize</td>
<td><em>Bipolaris maydis</em></td>
<td>Leaf blight</td>
<td>10—20</td>
</tr>
<tr>
<td></td>
<td><em>B. turcicum</em></td>
<td>Leaf blight</td>
<td>10—20</td>
</tr>
<tr>
<td></td>
<td><em>Fusarium moniliforme</em></td>
<td>Kernel rot</td>
<td>10—30</td>
</tr>
<tr>
<td>Crucifers</td>
<td><em>Alternaria brassicae</em></td>
<td>Leaf spot</td>
<td>5—20</td>
</tr>
<tr>
<td></td>
<td><em>A. brassicola</em></td>
<td>Leaf spot</td>
<td>5—20</td>
</tr>
<tr>
<td></td>
<td><em>Sclerotinia sclerotiorum</em></td>
<td>Stalk rot</td>
<td>5—15</td>
</tr>
<tr>
<td></td>
<td><em>Xanthomonas campestris pv. campestris</em></td>
<td>Black rot</td>
<td>10—30</td>
</tr>
<tr>
<td>Soybean</td>
<td><em>Cercospora kikuchi</em></td>
<td>Purple seed stain</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Colletotrichum dematium</em></td>
<td>Anthracnose</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Soybean mosaic virus</em></td>
<td>Hilum bleeding</td>
<td></td>
</tr>
</tbody>
</table>
With the increasing distribution of germplasm between researchers and countries, it is important that germplasm banks and international trade do not harbour and distribute seed-borne pathogens, as fungal pathogens can travel on, in or with seed batches. Major plant diseases that have been spread around the world through trade (though not necessarily in seed) include powdery mildew (on grapes), citrus canker, potato wart, wheat flag smut and Dutch elm disease. In order to reduce such transmission routes, safeguards have been introduced to prevent the spread of pests and pathogens while allowing safe, uninterrupted seed movement. These safeguards vary widely from country to country, but include rules and regulations, import permits, phytosanitary certificates, inspections, treatments, isolation, passage through quarantine greenhouses and seed health testing. Seed health tests seek to establish the presence of a pathogen in the seed and to make an estimate of the extent of this infection within the seedlot (Reeves, 1995).

**Table 14.3.** Important seed-borne fungal pathogens in Nepal (Manandhar *et al.*, 1992).

<table>
<thead>
<tr>
<th>Genus of pathogen</th>
<th>Total species</th>
<th>Total hosts</th>
<th>Pathogen species of economic importance</th>
<th>Host</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alternaria</em></td>
<td>11</td>
<td>35</td>
<td><em>A. brassicae</em> and <em>A. brassiacola</em></td>
<td>Crucifers</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>A. padwickii</em></td>
<td>Rice</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>A. triticina</em></td>
<td>Wheat</td>
</tr>
<tr>
<td><em>Aspergillus</em></td>
<td>2</td>
<td>17</td>
<td><em>A. flavus</em> and <em>B. oryzae</em></td>
<td>Groundnut, maize</td>
</tr>
<tr>
<td><em>Bipolaris</em></td>
<td></td>
<td></td>
<td><em>B. sorokiniana</em></td>
<td>Rice</td>
</tr>
<tr>
<td><em>Botryodiplodia</em></td>
<td>1</td>
<td>1</td>
<td><em>B. theobromiae</em></td>
<td>Maize</td>
</tr>
<tr>
<td><em>Botrytis</em></td>
<td>1</td>
<td>3</td>
<td><em>B. cinerea</em></td>
<td>Chickpea</td>
</tr>
<tr>
<td><em>Cercospora</em></td>
<td>2</td>
<td>2</td>
<td><em>C. kikuchii</em></td>
<td>Soybean</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>C. oryzae</em></td>
<td>Rice</td>
</tr>
<tr>
<td><em>Colletotrichum</em></td>
<td>3</td>
<td>5</td>
<td><em>C. capsici</em></td>
<td>Chilli</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>C. lindenuthianum</em></td>
<td>Beans</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>C. truncatum</em></td>
<td>Soybean</td>
</tr>
<tr>
<td><em>Diplodia</em></td>
<td>1</td>
<td>1</td>
<td><em>D. maydis</em></td>
<td>Maize</td>
</tr>
<tr>
<td><em>Drechslera</em></td>
<td>12</td>
<td>16</td>
<td><em>D. tritici-repentis</em></td>
<td>Wheat</td>
</tr>
<tr>
<td><em>Fusarium</em></td>
<td>9</td>
<td>33</td>
<td><em>F. moniliforme</em></td>
<td>Rice and maize</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>F. oxysporum</em></td>
<td>Legumes</td>
</tr>
<tr>
<td><em>Phoma</em></td>
<td>1</td>
<td>9</td>
<td><em>P. glumarum</em></td>
<td>Rice</td>
</tr>
<tr>
<td><em>Protomyces</em></td>
<td>1</td>
<td>1</td>
<td><em>P. macrosporus</em></td>
<td>Coriander</td>
</tr>
<tr>
<td><em>Pyricularia</em></td>
<td>2</td>
<td>2</td>
<td><em>P. oryzae</em></td>
<td>Rice</td>
</tr>
<tr>
<td><em>Tilletia</em></td>
<td>3</td>
<td>2</td>
<td><em>T. indica, T. laevis</em> and <em>T. tritici</em></td>
<td>Wheat</td>
</tr>
<tr>
<td><em>Ustilaginoidea</em></td>
<td>1</td>
<td>1</td>
<td><em>U. virens</em></td>
<td>Rice</td>
</tr>
<tr>
<td><em>Ustilago</em></td>
<td>3</td>
<td>3</td>
<td><em>U. maydis</em></td>
<td>Maize</td>
</tr>
</tbody>
</table>
14.2 Problems Encountered in the Study of Seed-borne Disease

Imported seed and the transport of plant varieties from one geographical region or ecological zone to another is increasing, and this practice may introduce new diseases where they had not been encountered previously. Seed-borne inoculum may give rise to progressive disease development in the crop, and symptoms of disease may not be apparent immediately in the germinating seed. The causes of poor germination are often not identified by farmers and this is further complicated by the fact that the incidence of fungal infection of seed is not necessarily translated into subsequent incidence of disease. Seed that is infested with a fungal pathogen may be discoloured and poorly filled and thus have a poor market value, and seed that appears healthy may harbour low populations of the pathogen that later serve as an inoculum source. Little work has been undertaken to date to determine the relative significance of different sources of inoculum in disease development for even the most widely grown crops. It is, therefore, important to: (i) understand the structure of fungal populations; (ii) determine whether individual strains can be identified; (iii) determine how quickly the pathogenic strains adapt to their plant hosts; (iv) determine how rapidly they might adapt to changes in the cultivars grown; and (v) ascertain whether alternative hosts are significant to the life cycle or to disease dissemination. There are already relatively straightforward methods to differentiate between different species in a pathogen complex but it remains particularly difficult to differentiate between virulent and non-virulent strains of the same pathogen species.

14.3 Methods Available in the Study of Seed-borne Disease

Traditional methods in the recognition and detection of seed-borne organisms include: (i) direct observation; (ii) microscopic examination of imbibed seeds; (iii) examination of cultured organisms removed by washing; (iv) examination of seed after incubation (including the blotter test and the agar plate test); and (v) examination of growing plants (‘growing-on’ test). Each of these requires three steps: extraction, isolation and identification (Reeves, 1995). Such methods can be adapted to discriminate between particular species through exploitation of nutritional requirements. For example, Gnanamanickam et al. (1994) used selective media to detect Xanthomonas oryzae pv. oryzae in rice seed. However, despite the semi-selectivity of the media chosen, the sensitivity of the seed assay remained inadequate because even the faster growing X. o. oryzae strains were not recovered unless present in relatively high concentrations (2 × 10^5 to 1 × 10^6 c.f.u. ml⁻¹) in the seed extract. Detection of seed-borne X. o. oryzae on semi-selective media has been a challenge because the pathogen grows very slowly, and growth is
suppressed by contaminating bacteria found on seed. The more traditional methods suffer the disadvantage that they are slow and the sensitivity and accuracy of diagnosis can be a problem.

The last decade has seen great advances in diagnostic technology, especially in the development of rapid and sensitive methods (Pearce and Holderness, 1996; Richardson, 1996). Recently, there has been a change in emphasis from a more traditional morphological approach to identification of plant pathogens (for example, spore size, shape and colony colour) to a more functional approach based on aspects of the life cycle (for example, mechanism of spore production, DNA relationships and physiological attributes). This has come about firstly through immunodetection techniques (including polyclonal antisera and monoclonal antibodies, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence microscopy), and then methods currently in use and under development for the differentiation of different strains of filamentous fungi, which include the use of secondary metabolites, mycotoxins/antibiotics, protein electrophoresis, isoenzyme electrophoresis, fatty acid analysis (Paterson and Bridge, 1994) and DNA-based technology. DNA-based methods have included restriction fragment length polymorphism (RFLP) and pulse-field gel electrophoresis (PFGE) methods (Kasuga et al., 1993), and PCR amplification of various genome regions including rRNA genes and random amplified polymorphic DNA (RAPD) (Schots et al., 1994).

Ball and Reeves (1991, 1992) reviewed the use of ELISA, PCR, RAPD and similar methods to identify pathogens on seed, but the effort to date has been concentrated on the detection of bacteria and viruses. There is currently much effort being devoted to the further development of such techniques; Slack et al. (1996) have prepared a comparison of PCR, ELISA, and DNA hybridization for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes. Seed-borne organisms, however, can provide particular problems not necessarily encountered with organisms in pure culture. For example, RAPD cannot be used directly to detect seed-borne organisms because of non-specific hybridization of the primer which may occur with extraneous DNA from seed extracts (Reeves, 1995), and although PCR techniques have been used for the characterization and identification of fungi, little attention has been given to the detection of seed-borne pathogens (Reeves, 1995). Lange (1986) examined nucleic acid techniques for the detection of seed-borne viruses, and their use for the detection of plant pathogenic bacteria (Rasmussen and Reeves, 1992) and seed-borne diseases (Reeves, 1995) has been reviewed. Blakemore et al. (1994), by testing fungi isolated from seeds, noted that such methods are not yet at a stage for incorporation into seed health tests, but because of their high sensitivity, these techniques will continue to be used to detect microorganisms which are difficult to culture or difficult to identify.
14.4 PCR Methods Available for the Study of Seed-borne Diseases

The ability to amplify DNA from crude mycelial preparations is an important factor in the identification of filamentous fungi from plant material, as is the ability of the PCR reaction to use small amounts of material and even partly degraded materials of poor quality. Due to these features many potential sources of fungi can be used, such as unpurified genetic material or material taken directly from soil, growing mycelia, museum specimens and spores (Foster et al., 1993). The first stage in the analysis of seed-borne organisms is the culture of the organism from the seed; this is necessary, because PCR can detect dead bacteria and fungi which would not be a problem in seed. Where only one organism is present, or where a species-specific test is to be used, this can be undertaken in small volumes (500 µl) of a rich liquid growth medium over 72 h at 30°C. DNA can then be extracted using a range of different techniques including that of rapid extraction (Cenis, 1992; Raeder and Broda, 1985; Zolan and Pukilla, 1986). Specific DNA fragments can be amplified by PCR and products separated by gel electrophoresis. Banding patterns can be used to distinguish between species or varieties (Ball and Reeves, 1991). PCR can be carried out with RAPD primers (Williams et al., 1990); these are very short primers, not having identified DNA sequences, and usually 8–12 bases long (Caetano Anolles et al., 1992) which will hybridize to a large number of arbitrary sites in the genome (Fox, 1993). RAPD bands aid identification where this is primarily difficult. Examples are shown in Figs 14.1 and 14.2.

Higher specificity than that obtained with RAPD can be achieved by using variable non-translated repeat (VNTR)-derived oligonucleotides as PCR primers. This molecular method for differentiating fungal populations, and for differentiating between isolates, has been widely established and tested in the field (for example, see Bridge et al. 1997 and Pearce et al. 1996). Representative oligonucleotide primers can be selected to give either a high or a low discrimination between strains. Amplification products obtained from a range of isolates can then be used to group different strains of the same species. Following initial work with Sarocladium oryzae and Bipolaris oryzae (Fig. 14.3), the method has been tested against other common rice pathogens including Gerlachia oryzae, Fusarium moniliforme, Pyricularia oryzae and Rhizoctonia solani (Bridge et al., 1997; Pearce et al., 1996).

PCR-based methods have also been used in the investigation of air-borne spread of fungal pathogens. For example, a PCR-based method has been developed for the identification of Tilletia indica, the causal agent of Karnal bunt of wheat (Smith et al., 1996). Oligonucleotide primers were designed from a sequence derived from cloned DraI fragments of mitochondrial DNA and were used to analyse teliospores which had been germinated from a seed wash extraction method of infested grain. The results demonstrated
that *T. indica* could be reliably detected at an infestation level of five teliospores per 50 g grain.

Fig. 14.1. Banding patterns obtained from *Sarocladium oryzae* from RAPD primers. 
*Panel A*: the first nine horizontal lanes show banding patterns for DNA from nine different strains amplified using primer A11. The next ten lanes show patterns from ten isolates amplified using primer no. 71. The last lane shows the molecular size markers. 
*Panel B*: the first nine horizontal lanes show DNA from nine isolates amplified using primer A13, lane 10 shows one isolate amplified using primer A14, and lane 11 shows one isolate using primer A13. The next seven were amplified using primer A14. The last lane shows the molecular size markers.
14.5 Applications of PCR in the Analysis of Seed-borne Disease

14.5.1 Fungi

The utility of PCR as a specific and sensitive assay for plant pathogen identification is well documented (Henson and French, 1993; Smith et al., 1996). PCR and its applications in fungal disease diagnosis have also been described on many occasions (for example, Annamalai et al., 1995). PCR has also been shown to be a useful tool in the assessment of seed-borne inoculum, in measuring seed-borne inoculum potential, the extent of transmission from seed to crop, the extent and intensity of disease in the crop and seed-borne inoculum in relation to yield reduction and crop losses (Pearce and Holderness, 1996). PCR methods have been used in numerous other studies for identifying fungal pathogens from seed, including the
detection of *Pyrenophora* species, *Fusarium moniliforme*, *Stenocarpella maydis*, and the *Phomopsis/Diaporthe* complex (see Blakemore et al., 1994). A PCR-based method has been developed for the identification of *Tilletia indica*, causal agent of Karnal bunt of wheat, in seed wash (Smith et al., 1996) and to develop an assay to detect *Fusarium poae* in wheat (Parry and Nicholson, 1996). The latter assay was highly sensitive; it was capable of detecting *F. poae* at less than 1% seed infestation and it overcame problems of conventional isolation methods such as competition from other fungi. The key advantage here is that the method can detect specific pathogens in the presence of more aggressive species.

**Fig. 14.3.** Eight isolates of *Sarocladium oryzae* and eight isolates of *Bipolaris oryzae* tested with VNTR primers. Panel A: Primer GF, *S. oryzae* lanes 1–3, 7–9 and 11. *B. oryzae* lanes 4 and 5 and 12–17. Size markers are shown in lane 6. Panel B: Primer RY, *S. oryzae* lanes 1, 5 and 6. *B. oryzae* lanes 2–4, 7–11 and 17. Size markers are shown in lane 2.
14.5.2 Other pathogens

PCR has been used in studies identifying other pathogens from seed including the detection of *Pseudomonas syringae* pv. *pisi* (Rasmussen and Wulff, 1990), the halo blight bacterium *Pseudomonas syringae* pv. *phaseolicola* in phaseolus bean seeds (Prosen *et al*., 1991; Tourte and Manceau, 1991), and in the recognition and detection of the *Xanthomonas* pathogens that cause cereal leaf streak in seed.

Maes *et al*. (1996) have suggested that PCR-based techniques could be useful for the identification of bacteria in seed, and Bariana *et al*. (1994) have developed a method for detection of five seed-borne legume viruses in one sensitive multiplex PCR test. PCR has been used to develop a rapid and sensitive colorimetric detection of *Xanthomonas axonopodis* pv. *citri* by immunocapture and a nested-PCR (Hartung *et al*., 1996).

14.6 Limitations of the PCR Method

The PCR method has a great deal of potential in seed pathology, but there are also a number of problems and limitations associated with its use. Limitations of PCR reactions described elsewhere in this book will also apply to the study of seed pathology. In particular, there are a number of variables within the PCR itself which must be optimized for each primer–species combination. The dNTP concentration must be optimized and increasing the MgCl₂ concentration from 1 to 10 mmol l⁻¹ can have a dramatic effect on the specificity and yield of the amplification (Saiki, 1990). Studies with *Bipolaris* and *Sarocladium oryzae* isolated from rice seed *Oryza sativa* have shown that there can be both an increase and a decrease in the number of VNTR bands produced when the annealing temperature is raised from 35 to 47°C (Bridge *et al*., 1997; D.A. Pearce unpublished work). High primer concentrations can promote mispriming and the accumulation of non-specific PCR products when different buffers are used. Other variables which require optimization include cycle temperatures and durations, primer concentration and sample concentration and purity. Such problems are compounded when the reaction mixture becomes more complicated, for example, Bariana *et al*. (1994) stated that the multiplex assay was less robust and required very specific conditions. As several factors all affect the specificity and efficiency of DNA amplification, optimization is essential. No single protocol will be suitable for all applications, and each new PCR application will require optimization.

The problems associated with PCR reactions in terms of consistency and accuracy of consecutive reactions require the preparation of adequate controls for each PCR run. Such controls should include a strain with a known banding pattern (from a previous run) and an internal positive control to ensure that a negative result is not the result of failure of the PCR reaction, such as may be caused by inhibitors in the sample extract. It is also important
in seed pathology to conduct some form of pathogenicity test to back up the data. This is important, as the PCR reaction works equally well for dead and for living material, and so may amplify plant DNA. Virulence analysis and pathogenicity testing can also determine whether the increased occurrence of a particular strain is due to increased transmission in the seed or to a higher virulence. Care must be taken to prevent extraneous DNA from contaminating the reaction. All stock solutions must be fresh and sterile, and the possibility of carry-over contamination must be minimized.

Interference may occur at two stages of the strain analysis, either at the extraction stage when the fungus is isolated from the seed or in the PCR reaction mixture. An example of interference at the extraction stage is given by Gnanamanickam et al. (1994) who experienced problems in the detection of *Xanthomonas oryzae* pv. *oryzae* in rice seed. In this case direct isolation from seed is difficult, because *X. o. oryzae* grows slowly on media and does not compete with faster growing contaminant bacteria that may show similar colony characteristics and pigmentation. An example of interference at the amplification stage is where the PCR method is most effective with purified DNA but also works with crude preparations; crude DNA samples from seed may contain large amounts of extraneous DNA and this could result in considerable non-specific hybridization with primer DNA (Vivian, 1992). During multiplex PCR, it is important to select primers which do not anneal to each other; where a mixture of genomic DNA from two different fungi is used as template for different primers, competition between the two amplicons can cause inconsistent results. Chemicals from the environment, or other components of the seed/plant tissue, may interfere with the PCR. Hartung et al. (1996) found that a nested PCR improved the sensitivity from the single-stage PCR approximately 50- to 160-fold. However, they also found that the addition of citrus extracts and copper (used in leaf sprays) inhibited nested PCR amplification of *Xanthomonas axonopodis* pv. *citri*.

A number of studies have been conducted to determine the reproducibility of PCR in differentiating fungal populations. For example, Slack et al. (1996) compared PCR, ELISA and DNA hybridization for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes. They found that the results of each assay were significantly affected by inoculum dose, cultivar and sampling date. Of the inoculated samples 36.2, 35.8 and 29.1% tested positive by PCR, ELISA and DNA respectively.

### 14.7 Further Method Development

A current major disadvantage in the use of nucleic acid methods in seed health testing is that of quantification (Reeves, 1995) because it is not yet possible to determine the number of infected seeds in a batch, and there is a need to link presence *per se* with a measurement of potential future disease incidence. Colhoun (1983) has considered the need to be able to measure the
inoculum load on individual seeds in order to calculate threshold levels for seed health tests.

The choice of primers is important and can be improved, as it is suggested that primers with longer sequences of bases (up to about 20) are less likely to be present at random, even in the large genomes of plants (Fox, 1993) and will, therefore, achieve specific hybridizations.

Numerous minor manipulations can be made to the reaction process, in order to increase processing speed and to increase accuracy. For example, the use of cell lysates instead of extracted DNA obviates the DNA extraction step, inactive primers which are activated by the first denaturation step prevent primer dimers from forming and computer analysis packages, for example GELCOMPAR, can speed interpretation of results.

Vera-Cruz et al. (1996) have used repetitive extragenic palindromic (REP)-PCR and RFLP analysis to measure haplotypic variation in *X. oryzae* pv. *oryzae* within a single field. Their technique involved the analysis of the genomic structure of the field population by repetitive sequence-based PCR, with oligonucleotide primers corresponding to interspersed repeated sequences in prokaryotic genomes and RFLP with the insertion sequence IS1113. REP-PCR involves the use of specific primers (BOX, ERIC, ERIC2, REP and REP2). Competitive PCR may allow some degree of quantification of pathogens present in seed batches (Nicholson et al., 1993).

Amplified fragment length polymorphism (AFLP) may provide greater specificity and reliability, as would DNA probes that can be obtained by excising species-specific bands from a gel (Blakemore et al., 1992). Specific primers, obtained by sequencing such a band, could be used to develop a PCR-based test and provide a means of accurate identification and sensitive, rapid detection.

The internal transcribed spacer (ITS) of the ribosomal DNA (rDNA) subunit repeat was sequenced in representative isolates of *Cylindrocladium floridanum* and *Cylindrocarpon destructans* (Hamelin et al., 1996). These sequences were aligned and compared with ITS sequences of other fungi in the GenBank database. Two internal primers were then synthesized for the specific amplification of portions of the ITS regions of each of the species. These products were then used to develop a test in which species-specific fragments were amplified directly from infected roots from which one or two of the fungi had been isolated. ITS regions evolve rapidly, and may vary among species within a genus or among populations.

Nested PCR, in which a second round of PCR using primers internal to those of the first round is performed (Schesser et al., 1991), may be used to overcome non-specific amplification products from other organisms and from host tissues. Multiplex PCR would allow simultaneous testing for more than one organism by the use of mixtures of primers which are specific for individual organisms. Ligase chain reaction (LCR), like PCR, is a DNA amplification system that is highly specific and very sensitive (Lee, 1993) and even a single mismatch can prevent the reaction. High specificity has,
however, meant that it has not yet been used in plant pathology (Reeves, 1995).

14.8 Future Potential for the Study of Seed-borne Disease

There is a wide range of potential applications of PCR methodology in the analysis of seed-borne disease transmission. The potential exists for the use of the PCR method with washings from seed samples (Vivian, 1992) and without the need for extraction and isolation of the organism (Ball and Reeves, 1991). The revision of species concepts in individual pathogen populations is also being enhanced by the use of such molecular techniques.

Factors limiting the transmission of seed-borne disease can be enhanced in order to reduce disease transmission and enhance yield. In order to do this, it is important to know exactly what influence the alteration of cultural practices is having on the seed-borne element of transmission. These factors could include the impact of climate, storage, seed crop management, location of production, adjustment of cultural practices, chemical protection, protective inoculation, seed treatment and seed health testing (Misra et al., 1995). Disease management can involve the adjustment of sowing dates, deep ploughing, balancing host nutrition, intercropping, management of pollen and removal of collateral hosts. Control and monitoring of seed exchange can prevent the spread of pathogens limited to a small area. Other parts of the cropping cycle which can be exploited for disease management include: storage practices, the number of crops grown per year or the order of crops, climate, planting method, seed treatment and plant density.

PCR is, therefore, a useful tool for the investigation of seed-borne disease. PCR can be used in plant pathology to understand the structure of fungal populations, of both pathogens and their non-pathogenic antagonists, determine whether individual races exist; for example, is the Sarocladium oryzae that infects rice (Oryza sativa) the same pathogen that attacks Bamboo (Bambusa spp.) or is it a different species? (See Bridge et al., 1989.) PCR methods may allow pathologists to determine how quickly a particular pathogen is evolving, or adapting to a new host or cultivar, and whether alternative hosts are significant to the pathogen life cycle or disease dissemination.

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15.1 Introduction

Polymerase chain reaction (PCR)-based amplification of target nucleic acids was conceived by Kary Mullis in California in 1983 (Mullis, 1990). To date it is estimated that since the first publication in 1985 there have been over 40,000 references to PCR (White, 1996). Over the last decade PCR has become one of the most important and powerful tools in molecular biology. Two crucial technological developments – the introduction of thermostable DNA polymerases and automated thermal cyclers – have led to the common use of PCR for research and diagnostic purposes. Considerable effort is underway to make the PCR technology more robust, cost effective and user friendly.

In this chapter, some of the recent developments in amplification and detection technologies, the currently available diagnostic PCR kits, future applications of PCR in mycology and perspectives are discussed. A number of PCR based applications (Table 15.1) are currently used routinely by many laboratories and will not be covered in detail here. Further information on these techniques can be obtained from other reviews (Erlich and Arnheim, 1992; Foster et al., 1993).

15.2 Molecular Ecology, Epidemiology and Strain Typing

Integration of genetic markers with biological traits has helped to address questions on mating systems, origin of strains, gene flow, and host–parasite and symbiotic interactions. RFLP-based markers have been used extensively
for this purpose. Recently, PCR technology has enabled rapid strides to be taken in this area. PCR-based multilocus genotyping is likely to resolve questions such as the enhanced virulence or transmissibility of strains and also the complexity of the pathogen population of a disease outbreak. For example, multilocus genotyping (arbitrarily primed PCR) of the asexual human fungal pathogen *Coccidioides immitis* revealed that the haploid organism was completely recombining, within geographically isolated populations, even though no sexual stage had been recorded (White, 1996). Such data can be used to trace the geographic origin of an infection/pathogen. In agricultural ecosystems such high resolution genetic information will be important to determine whether sexual recombination and gene flow are occurring regularly during a pathogen’s life history. Reassortment of the virulence genes, leading to new combinations, could very quickly overcome genes introduced for resistance. PCR is also likely to negate the limitations imposed on our understanding of microbial diversity using traditional microbiological methods. A number of PCR-based techniques suitable for these applications are emerging.

### Amplified fragment length polymorphism (AFLP) analysis

AFLP is a tool that allows differentiation between individuals, genotypes and strains and the assessment of genetic diversity and phylogeny. Originally developed for genetic mapping in plants (Vos *et al.*, 1995), this technique has the characteristics of an ideal system for detecting genetic variation.

The AFLP markers generated are ‘neutral’ (i.e. not subject to natural selection) and are generated from a large number of independent loci from different parts of the genome. The profiles generated are reported to be highly reproducible, because of the high stringency PCR due to the nature of the primers (Majer *et al.*, 1996; Mueller *et al.*, 1996).

AFLP analysis involves selective amplification of fragments from restriction enzyme digests of genomic DNA. Approximately 500 ng of genomic DNA is digested simultaneously with a hexanucleotide-cutter *Eco*RI and a tetranucleotide-cutter *Mse*I. This results in more than 150,000 fragments from a fungal genome of approximately $5 \times 10^7$ bp. The digested

<table>
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<th>Table 15.1. Present applications of PCR in mycology.</th>
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<tr>
<td>RAPD–PCR</td>
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<td>RT–PCR</td>
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<tr>
<td>Inverse PCR</td>
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<tr>
<td>PCR mutagenesis</td>
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<tr>
<td>PCR gene construction</td>
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<td>PCR SOEing*</td>
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*Splicing by overlap extension.
fragments are ligated with an MseI adaptor and a biotinylated EcoRI adaptor. Fragments with the EcoRI adaptor are captured using streptavidin beads for PCR. The numerous, small Mse–Mse fragments are discarded. Primers are designed to match each of the restriction enzyme adaptor sequences with the addition of an arbitrary two base extension at the 3′ ends and PCR is then performed at high stringency (Fig. 15.1). This leads to the amplification of a manageable number of fragments. Usually 50–70 fragments can be resolved from one PCR on denaturing polyacrylamide gels and the whole procedure can be completed in 2 days. Ready-to-use kits (e.g. Life Technologies, Perkin Elmer/Applied Biosystems) are available for AFLP analysis.

15.2.2 Long PCR

Restriction fragment length polymorphisms (RFLPs) in rDNA and mtDNA have been used extensively in assessing genetic diversity and species relationships in various fungi. Conventionally, polymorphisms are detected by Southern hybridization of the digested genomic DNA samples with a specific probe (e.g. Sreenivasaprasad et al., 1992). RFLPs from PCR amplified segments (up to 2.0–2.5 kb) of rDNA have also been used in similar studies (e.g. Muthumeenakshi, 1996).

Recent development of the ‘long PCR’ technique, which employs two DNA polymerases (non-proofreading Taq and a proofreading Pwo) has led to the amplification of 10–40 kb fragments. This has opened up new possibilities for the rapid generation of RFLPs and mapping.

Amplification of full-length animal mitochondrial DNA by long PCR, by using a set of forward and reverse primers from the 16S RNA gene, has recently been reported (Nelson et al., 1996). The size of mtDNA in animals ranges from 16 to 20 kb. However, amplification of full-length mtDNA from fungi is likely to be more challenging because the size of mtDNA in fungi varies between 20 and 170 kb (Taylor, 1986). Nonetheless, using appropriate primers, large fragments can be amplified by long PCR to generate RFLPs. Fragments of rDNA of up to 7 kb have been amplified from *Trichoderma* spp. with universal ribosomal primers (White et al., 1990) in long PCR, and used to detect RFLPs (S. Muthumeenakshi, Warwickshire, 1996, personal communication).

A rapid vector-independent restriction mapping technique has also been developed using long PCR. PCR primers are radioactively labelled to serve as probes for DNA fragments generated by partial digestion of the PCR product. This method has been used to generate restriction maps of 8–18 kb fragments directly amplified from human genomic DNA (Her and Weinsilboum, 1995).

Another application of long PCR is in repetitive-element based PCR (REP-PCR). Dispersed repeat sequences in *Magnaporthe grisea* are being used extensively to genotype the isolates by Southern hybridization-based fingerprinting (Levy et al., 1991; Xia et al., 1993). Recently, REP-PCR has
Fig. 15.1. Amplified fragment length polymorphism (AFLP) technique.
been developed for genotyping *M. grisea* isolates (George *et al.*, 1997). Two outwardly directed primers, designed from a repetitive element sequence, were used in combination with long PCR to generate distinct banding patterns (Fig. 15.2) with *M. grisea* isolates from different hosts. The amplicons ranged in size from 0.4 kb to longer than 23 kb, although most scorable bands were less than 23 kb in size. The PCR profiles generated differentiated the rice-infecting and rice non-infecting *M. grisea* isolates. Robust groupings and close correspondence between the *Pot2* REP-PCR and MGR586 RFLP lineages were observed upon cluster analysis of PCR profiles from clonal as well as highly diverse rice-infecting *M. grisea* populations.

![Flowchart showing the REP-PCR technique.](image)

**Fig. 15.2.** Flowchart showing the REP-PCR technique.
15.3 Diagnostic PCR

PCR-based detection of pathogenic fungi directly from infected tissue has been reported for several important systems (Lévesque et al., 1994; Sreenivasaprasad et al., 1996). PCR has also been used to monitor mycorrhizal symbionts (Henrion et al., 1994) and to detect pathogens directly from soil (Henson et al., 1993). Recently, a nested multiplex PCR approach has been developed to simultaneously detect conifer root rot fungi Cylindrocarpon destructans and Cylindrocladium floridum (Hamelin et al., 1996).

With most of these PCR methods, which are based on rDNA sequences, infected tissue with dead mycelium might also test positive as the DNA could still be amplified. It would be preferable to discriminate between viable and non-viable propagules of the pathogen, particularly in the context of certification programmes and quarantine tests. In order to detect the viable propagules only, it would be necessary to develop reverse transcriptase (RT)–PCR-based tests, using RNA as the source of amplification. Species-specific primers from rDNA sequences can be used in RT–PCR, although it is not certain whether amplification of ribosomal RNA can always be directly correlated with viable biomass. RT–PCR amplification of sequences such as chitin synthase genes and ergosterol biosynthetic genes can be more reliably related to the viability of the propagules. These RT–PCR tests based on gene sequences, however, are likely to be less sensitive as the abundance of these messages is much lower as compared with the ribosomal RNAs.

Currently, a number of diagnostic kits (Table 15.2) are available for routine use in forensic and clinical laboratories as a result of concerted research and development efforts between academia and industry, backed by strong funding. Similar advances can be envisaged for PCR application in mycology in biomedical fields.

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<thead>
<tr>
<th>Kit name</th>
<th>Basis of detection</th>
<th>Use</th>
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<tbody>
<tr>
<td>AmpliType PM</td>
<td>Five independently inherited genetic loci</td>
<td>Forensic testing and human identity</td>
</tr>
<tr>
<td>AmpliFLP D1S80</td>
<td>16 bp Repeat on chromosome one</td>
<td>Forensic testing and human identity</td>
</tr>
<tr>
<td>Enviroamp Legio.</td>
<td>5S Ribosomal RNA gene and macrophage Legionella infectivity potentiator gene</td>
<td>Detection of Legionella</td>
</tr>
<tr>
<td>Amplicor Myco.</td>
<td>16S Ribosomal RNA gene probe</td>
<td>Detection of Mycobacterium tuberculosis</td>
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<tr>
<td>Amplicor hep.</td>
<td>RT–PCR of viral RNA</td>
<td>Detection of hepatitis C virus</td>
</tr>
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</table>

Table 15.2. Some of the currently available forensic and clinical diagnostic kits (Graham, 1994; Whelen and Persing, 1996) as examples of the range of PCR applications.
15.3.1 Data processing

The expected improvements in diagnostic capability necessitate the availability of software packages suitable for different applications. User-friendly interactive systems that answer questions in an efficient and simple manner are likely to facilitate the introduction of diagnostics into the user’s environment.

15.4 Genome Mapping and Sequencing

PCR has contributed to rapid completion of physical maps of human and other genomes (Graham, 1994; Marx, 1995; Abramowitz, 1996). PCR was used as the basis for translating the various physical mapping markers into a common language of short fragments of single-copy DNA sequence called sequence-tagged sites (STTs). Generally, 200–500 bp of sequence data would define an STT. An STT is unique to that particular genome and can be specifically amplified by PCR. An STT detection PCR assay can be carried out using two primers (~ 20 bases) designed to be complementary to the two strands and at opposite ends of the sequence segment. Thus, a 100 kb clone can be mapped by determining the order of a series of STTs and measuring the distances between them. This information can be completely described in a database along with the conditions for the PCR assay (Olson et al., 1989).

PCR has also had a considerable effect on genetic mapping through the amplification of the short tandem repeats (STRs) which are stretches of tandemly repeated short nucleotide motifs present in most eukaryotic genomes (Kashi et al., 1997). These elements occur as interspersed repeats and may be found by screening a genomic library. PCR primers can be designed for STR amplification by subcloning and sequencing (Litt, 1994). These STRs can be used both as polymorphic markers for measuring the frequency of recombination within families and also to interrelate physical and genetic maps. Further, substantial progress has been made in the analysis of complementary DNA clones to generate a segment of sequence called an expressed sequence tag (EST) by PCR as part of the Human Genome Project (White, 1996). A large volume of EST sequences is available from various animal and plant systems e.g. *Arabidopsis* (Newman et al., 1994). ESTs are generated by partial sequencing on cDNA clones. Clones from randomly primed cDNA libraries or partial cDNA clones can be used to generate this information rapidly. ESTs are useful for the identification and isolation of new genes, the identification of coding regions in genomic sequences and for genome mapping. PCR methods have also been used to map ESTs to specific chromosomes (Adams et al., 1991).

The wider application and success of genome sequencing projects depend on the availability of rapid and cost-effective sequencing technology. As part of the *Arabidopsis* genome sequencing initiative, a microthermal
cycler has been developed with which 0.5 µl sample volumes and 40 s cycle times can be achieved (Marziali et al., 1997). The two strategies presently employed are shotgun sequencing and primer walking. However, both of these approaches have time-consuming procedures. Large scale template preparation, generation of redundant sequences and the assembly of contigs are some of the major disadvantages with the former. With the latter, primer synthesis can be expensive and slow and automation is more complicated. The use of presynthesized libraries of primers with different sequences has been proposed to eliminate the primer synthesis step (Studier, 1989). Individual short primers from such a library are either ligated (Szybalski, 1990) or assembled without ligation (Beskin et al., 1995) to make unique sequencing primers. More recently, a conceptually new technique known as differential extension with nucleotide subsets (DENS) has been developed (Raja et al., 1997).

15.4.1 Differential extension with nucleotide subsets (DENS)

DNA sequencing using DENS is a two stage process (Fig. 15.3). Initially, a short primer is allowed to extend at 20–30°C with only two out of the four dNTPs present in the reaction mix. Primer (a partially degenerate octamer or a hexamer) extension depends on how soon a base corresponding to the dNTPs not used in the reaction is present in the strand being synthesized. This leads to differential extension of the primer, at different sites, depending on the template sequence. The intended priming site, the primer and the two dNTP subsets are chosen to maximize the length of the primer at that site. Elsewhere in the template, at randomly located priming sites, the primer extensions are likely to be substantially shorter. Subsequently, a termination reaction is performed at higher temperature with all four dNTPs present, similar to normal cycle sequencing. The higher annealing and extension temperature permits a sequence pattern to be generated only from the maximally extended site. Differential extension products of the same primer are shorter to anneal and extend and do not produce the sequence pattern. Although, at present, a <10% failure rate of DENS is expected because of the superimposed non-target sequence, further optimization of the procedures and conditions is likely to improve the usefulness of this technique.

With the increasing interest in mapping and sequencing of fungal genomes, particularly *Aspergillus* and *Neurospora* (Bennett, 1997; Hamer, 1997), it is likely that PCR will have a major impact in this area.

15.5 Gene Expression

Various life processes, such as development, differentiation and response to stress, are controlled by regulation of gene expression. Identification and
Fig. 15.3. Differential extension with nucleotide subsets technique for sequencing.
isolation of the differentially expressed genes are essential to understand and manipulate these biological processes. Conventionally, differential hybridization of cDNA libraries has been used to isolate these genes. Recently, various techniques that combine the advantages of PCR have been developed.

**15.5.1 Differential display reverse transcription–PCR**

Differential display reverse transcription–PCR (DDRT–PCR) was developed for isolating tumorigenesis-related genes from mice (Zhang and Medina, 1993) and humans (Liang et al., 1992), and has also been applied in the analysis of tomato fruit ripening-related genes (Oh et al., 1995). The technique facilitates rapid isolation of differentially expressed genes via PCR without the construction and screening of cDNA libraries.

The total mRNA pool from a sample is reverse-transcribed into 12 subpopulations of cDNA using 12 different anchored oligo-dT primers (e.g. T₁₁AC). The cDNA subpopulations are then PCR-amplified using the same anchored primer and an arbitrary 10-mer. Additional cDNAs comprising each subpopulation are amplified by using alternative arbitrary primers (Fig. 15.4). The composite PCR profile is representative of the mRNAs contained in each population.

Radiolabelled amplification products derived from the same anchor/ arbitrary primer combinations with different samples are displayed in adjacent lanes on polyacrylamide gels, to locate the differentially expressed genes. The gene fragments are excised, eluted, cloned and sequenced to identify the gene. These fragments can also be used as probes on genomic/ cDNA libraries to clone complete genes.

DDRT–PCR is potentially faster than differential hybridization of cDNA libraries and initially gained wide attention. Despite this, some doubts have been raised as to the wider applicability of this technique as it might be biased for high copy number mRNA species and inappropriate where only a few genes might vary. A number of modifications have been made to the original protocol to optimize the technique, for example the use of longer primers to reduce the level of false positives, and to improve reproducibility. Several ready-to-use kits are now commercially available (e.g. Gene Hunter, Pharmacia).

**15.5.2 Suppression PCR**

This is a technique used to selectively amplify differentially expressed (target) cDNA fragments whilst simultaneously suppressing non-target amplification. Suppression PCR is specifically designed to equalize the abundance of messages within target cDNA to enable the isolation of even rare transcripts (Diatchenko et al., 1996).

Tester and driver double-stranded cDNAs are prepared from the two mRNA samples under comparison. Two tester populations are created with
Fig. 15.4. RNA differential display technique.
Fig. 15.5. Suppression PCR technique.

Continued opposite
different adaptors but the driver has no adaptors. The first hybridization between excess driver cDNA and each population of the tester cDNA results in equalization of message abundance and enrichment of differentially expressed sequences in the tester. The second hybridization between fresh driver cDNA and the two primary hybridization samples mixed together leads to the generation of differentially expressed gene templates for PCR.

Fig. 15.5 continued.
Using suppression PCR, only the differentially expressed sequences are amplified exponentially during a first round PCR. A second round PCR with nested primers results in reduced background and enrichment of target sequences (Fig. 15.5).

This technique requires only 0.5–2.0 µg of poly(A)+ RNA as starting material and is designed to amplify rare transcripts, typically the most difficult to obtain. Suppression PCR does not involve physical separation of single-stranded and double-stranded cDNA. Strategic design and use of adaptors suppress the amplification of most types of molecules including the molecules where panhandle-like structures are formed by the long inverted repeats. Suppression PCR can thus achieve greater than 1000-fold enrichment for differentially expressed cDNA (Clontech, 1995). A ready-to-use commercial kit is available from Clontech.

15.5.3 In situ PCR

This technique is used to amplify target DNA or RNA within intact cells, permitting the correlation of PCR results with cellular localization and morphology. Fixed cells or tissue are permeabilized and contained and PCR reagents are added to this. Thermal cycling through suitable parameters is followed by detection of PCR products using appropriate methods e.g. fluorescence microscopy. Lack of uniform equipment is perceived as the major problem in performing and reproducing in situ PCR. Techniques adapted for in situ thermal cycling, in particular, vary widely. With the availability of new in situ PCR systems (e.g. Perkin Elmer/Applied Biosystems) it is expected that this technique can be performed consistently, reproducibly and reliably (Perkin Elmer, 1994).

In situ PCR is an emerging tool in virology, cancer research and developmental biology. This technique is likely to be employed with various fungal systems. For example it has been applied in the study of arbuscular mycorrhizal (AM) fungi to confirm the presence of different alleles in AM fungal genes and to identify different nuclear populations within a single AM spore (Berta et al., 1996).

In the last few years, there has been an upsurge in understanding the molecular basis of differentiation and development in both pathogenic and beneficial fungi. Various PCR-based techniques are likely to be applied widely on a routine basis.

15.6 Amplification Technology

Rapid advances are being made in the miniaturization of the PCR instruments and also in reducing the time required for amplification. For example, by adapting PCR on to a microinstrumentation platform and using suitable detection chemistries in minute disposable chambers (0.5–50 µl), 30 cycles
can be completed in 10–20 min on a very small instrument (White, 1996). Similarly, the use of silicon chambers which require only 10 µl reactions and are heated resistively can reduce amplification time by an order of magnitude. Under these conditions 30 cycles may be completed in 7 min on a microfabricated prototype. Results for a variety of loci using these systems were comparable with the results from conventional PCR. This apparatus was about the size of a scientific calculator including power supply and control electronics and four 9 V batteries allowed 2.5 h of operation (Abramowitz, 1996). Such portable and convenient devices are likely to be used for on-field/on-site diagnosis.

Progress in the automation of nucleic acid extraction in combination with the advances in PCR technology are likely to lead to the availability of completely automated instrument systems for diagnostic clinics in the foreseeable future.

15.7 Detection Technology

A number of techniques (Graham, 1994) can be used to analyse PCR products (Table 15.3). Among these methods, scintillation proximity assay (Amersham), electrochemiluminescence (Perkin Elmer) and microwell assays (e.g. Roche) are suitable for analysing a large number of samples routinely.

Further progress is being made in developing novel amplicon analysis and detection methods. DNA can be bound to a 96-well polystyrene microtitre plate by non-covalent interactions e.g. by 500 mM NaCl or streptavidin coating, or immobilized probes can be used to capture biotinylated PCR products. The plate-bound amplicons can then be analysed by enzyme-assisted colorimetric detection which enables the simultaneous handling of numerous samples. A variation of this method is the use of a biotinylated primer and a modified triphosphate (digoxigenin-11-dUTP) in PCR (Fig. 15.6). Detection is achieved by capturing the biotinylated

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amplicons and using anti-digoxigenin antibody conjugated to alkaline phosphatase and suitable substrates (O’Donnell-Maloney et al., 1996). Furthermore, by marking a specific primer with a unique hapten, e.g. digoxigenin or fluorescein, each reaction can be detected using hapten-specific antibodies labelled with different enzyme reporters, such as alkaline phosphatase or horseradish peroxidase. This system permits the detection of two different targets from a single reaction by different colour reactions (Tobe et al., 1996).

Another method of amplicon detection is the use of a biotinylated primer and a fluorescently labelled primer in PCR. Streptavidin captured amplicons are denatured, the fluorescently labelled strand is hybridized to an array of immobilized probes on a glass slide and the hybridization pattern and polymorphisms are detected by fluorescence scanning (Fig. 15.7). An example of the use of this method is the identification of polymorphisms from exon 4 of the human tyrosinase gene (O’Donnell-Maloney et al., 1996).

Detection methods that utilize reporter dyes are being integrated into new ‘kinetic’ thermal cyclers which can monitor the products during amplification. It is expected that these combined instrument–reagent systems
will enable rapid generation of both qualitative and quantitative data with high throughput (White, 1996). Recently, a microvolume multisample fluorimeter with rapid thermal cycling has been developed by Idaho

Fig. 15.7. Detection method based on amplicon hybridization to probe assays.
Technology, Idaho, USA. This instrument combines rapid thermal cycling capability, and fluorescent monitoring by three different techniques (Wittwer et al., 1997a, b).

Recently, scientists at USDA (Madison, Wisconsin) and Perkin Elmer (California) have applied an automated TaqMan assay system to detect Tilletia indica (Berthier-Schaad et al., 1997). The TaqMan system relies on the ability of the 5′ nuclease activity of Taq polymerase. During amplification, the reporter dye from the 5′ end of the probe is released and the resultant increase in fluorescence is monitored in real time. Under optimal PCR conditions, increase in fluorescent signal above the threshold level, by the end of the amplification cycles, detects a sample as positive for T. indica. Preliminary results on the comparison of classical PCR and TaqMan with T. indica from ryegrass and other hosts yielded approximately 60% agreement. With improvements in reliability, routine use of similar assay systems can be envisaged.

15.8 Conclusion

Rapid advances are being made in both amplification and detection technologies to enable rapid and high throughput PCR analyses, and the number of techniques available is continually increasing. In view of these developments, PCR is likely to be one of the most widely used tools in mycology in the broad context of genes and genomics as well as diagnostics.

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