1 Polymerase Chain Reaction in Mycology: an Overview

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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

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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

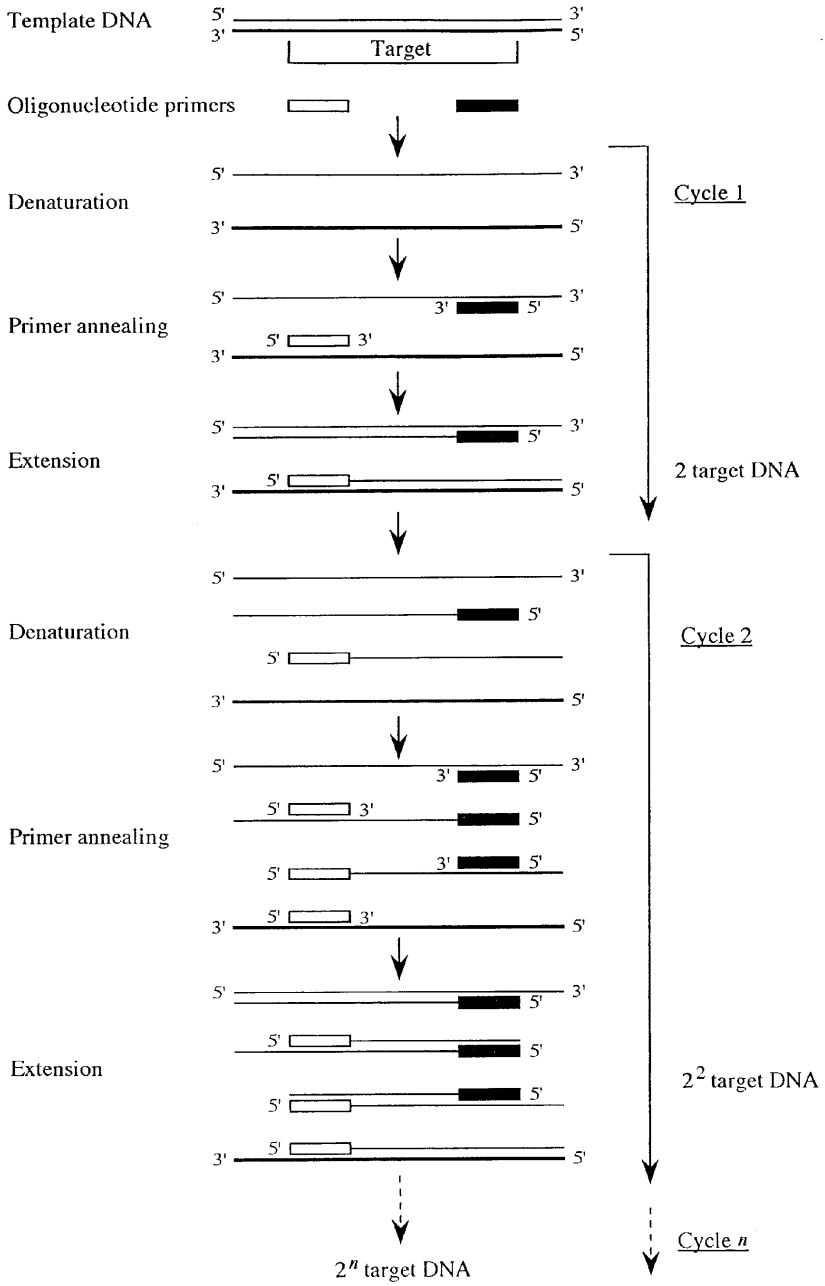

Fig. 1.1. Principle of PCR amplification.

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

Component	Concentration
Template DNA	10–100 ng
Amplification buffer	1/10 of final volume (buffer is supplied 10 × concentrated with the *Taq* DNA polymerase)
$MgCl_2$	0.5–5 mM (typically 1.5 mM)
dNTPs	20–200 µM each of dATP, dCTP, dGTP and dTTP
Primer 1	0.1–0.5 µM
Primer 2	0.1–0.5 µM
Taq DNA polymerase	0.5–2.5 units
Sterile distilled water	To final volume
Final volume	25–100 µl

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

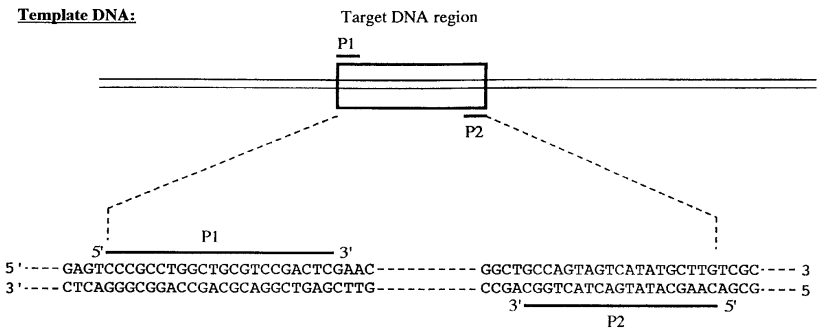

Fig. 1.2. Schematic representation of a target DNA region to be amplified by PCR and the sequence of the corresponding oligonucleotide primers.

(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 $3' \rightarrow 5'$ 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.

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