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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

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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 Stasovski, 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; Vigalys 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 *Ophiosphaerella 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 ITS-4B 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 populations 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 cinnamomi*, *Tuber magnatum* and *Glomus mosseae* (Dobrowolski and O'Brien, 1993; Lanfranco *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 systematics, 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; Mehmann *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 evoloutionary 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 comparitive 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 recent copying, 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 anlaysis 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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