Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens

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Advances in molecular technology show great potential for the rapid detection and identification of fungi for medical, scientific and commercial purposes. Numerous targets within the fungal genome have been evaluated, with much of the current work using sequence areas within the ribosomal DNA (rDNA) gene complex. This section of the genome includes the 18S, 5.8S and 28S genes which code for ribosomal RNA (rRNA) and which have a relatively conserved nucleotide sequence among fungi. It also includes the variable DNA sequence areas of the intervening internal transcribed spacer (ITS) regions called ITS1 and ITS2. Although not translated into proteins, the ITS coding regions have a critical role in the development of functional rRNA, with sequence variations among species showing promise as signature regions for molecular assays. This review of the current literature was conducted to evaluate clinical approaches for using the fungal ITS regions as molecular targets. Multiple applications using the fungal ITS sequences are summarized here including those for culture identification, phylogenetic research, direct detection from clinical specimens or the environment, and molecular typing for epidemiological investigations. The breadth of applications shows that ITS regions have great potential as targets in molecular-based assays for the characterization and identification of fungi. Development of rapid and accurate amplification-based ITS assays to diagnose invasive fungal infections could potentially impact care and improve outcome for affected patients.

Keywords fungal PCR, fungal ITS, internal transcribed spacer, rDNA gene complex

Introduction

The ability to determine the nucleic acid sequence of genomic DNA has revolutionized most areas of contemporary biomedical research. DNA and RNA have been used in clinical microbiology applications for the classification and identification of bacteria and eukaryotic pathogens, such as fungi for many years [1]. The ribosomal DNA (rDNA) genes are found in all microorganisms and known to accumulate mutations at a slow constant rate over time. Nucleotide sequence heterogeneity within this region can be used to phylogenetically classify microorganisms. Interspaced among the highly conserved sequences of the rDNA genes are regions of variable sequences called spacer regions. The function of these regions is not completely known; however, they are
historically referred to as spacers since they separate the functional DNA sequences of the various rDNA genes.

Since mutations within the spacer regions of the rDNA gene complex occur with greater frequency than with the rDNA genes, the sequence heterogeneity within this area has been useful for the separation of both genera and species. Early molecular studies showing the usefulness of the rDNA complex to classify bacteria were done by Gutel et al. [2]. In this group of microorganisms, the operon organization consists of a promoter region followed by a sequence coding for the 16S rDNA gene, a spacer (also referred to as the intergenic spacer), the 23S rDNA gene coding sequence, another short spacer, and finally the sequence coding for 5S rDNA gene. With the subsequent development of the polymerase chain reaction (PCR) technique in combination with this previous knowledge about the rDNA complex, techniques to identify bacteria using the 16S/23S ribosomal spacer as a target were developed [3,4]. This ability to amplify and to compare sequences within the 16S/23S-spacer region has had an enormous impact on the classification of bacteria [5,6]. Additionally, utilization of primers that recognize universally conserved sequences within the rDNA genes of prokaryotic cells which flank the variable signature sequence within the spacer region has allowed for the identification of pathogens which previously could not be cultivated [7–9].

Similarly, it is also recognized that eukaryotic cells such as fungi have a rDNA gene complex region with comparable characteristics. The organization of this complex in fungi includes a sequence coding for the 18S rDNA gene, an internal transcribed spacer region (ITS1), the 5.8S rDNA gene coding region, another ITS region (called ITS2) and the sequence coding for the 28S rDNA gene. As in the case of the 16S and the 23S rDNA genes, the coding regions of 18S, 5.8S and 28S nuclear rDNA genes evolved slowly, and are relatively conserved among fungi, providing a molecular basis of establishing phylogenetic relationships [10]. Between these rDNA gene-coding regions are the ITS1 and ITS2 regions, which are similar to the spacer regions in the bacterial rDNA that evolved more rapidly, leading to sequence variability among genera and species of fungi.

Early work in molecular testing using the rDNA complex as a target concentrated in the region of the 18S rDNA gene (also referred to as the small-subunit rDNA gene or the 16S-like rDNA gene) [11–16]. Other genes within the rDNA complex have also been used for the molecular evaluation of fungi. These include the 5S rDNA gene [17], the 5.8S rDNA gene [18] and the 28S rDNA gene (also referred to as the large-subunit rDNA gene, the 25S to 27S rDNA gene, or the 28S-like rDNA gene) [19–22]. The comparison of nucleotide sequences within these gene regions has been successful for the separation of fungal genera and species. However, limited sequence variability within these rDNA genes, together with a need to compare large sequence regions, has led to a shift to evaluate the shorter spacer regions as targets to separate fungal species.

The dramatic increase in the incidence of opportunistic fungal infections along with the development of new antifungal agents with various spectra of activity and the emergence of antifungal resistance has led to a critical need for diagnostic methods that can rapidly and accurately identify fungal pathogens [23–25]. The reported molecular techniques, using areas within the rDNA gene complex as a target, have shown promise for the detection and identification of fungal pathogens. The purpose of this paper is to review amplification methods that use ITS regions as molecular targets to diagnose and classify fungal pathogens. The commercial and industrial approaches using the ITS regions as targets have been evaluated in numerous other studies and will only be discussed in limited detail in this review [26–36]. Finally, for a more thorough discussion of PCR methods for the diagnosis of invasive fungal infections, the reviews of Reiss et al. [37] and Walsh and Chanock [38] are recommended.

**Biology of the ITS regions**

*Introduction*

A fascinating feature of biological life is the common use of the DNA genetic code and its subsequent processing into functional units of protein through the intermediate RNA molecule. The transcription of DNA into RNA and translation of RNA into protein are both highly regulated and compartmentalized in all living organisms. The cellular factory responsible for the production of protein is the ribosome. As the essential functions of ribosomes are critical for survival, their physical parameters have been conserved in all forms of life, from bacteria to humans. Some components within the ribosomal factories have, however, changed during the evolutionary process. These similarities, as well as the changes within genetic material can be used as tools for the identification of microorganisms including fungi, which is the focus of this review. The sequence homology within the rDNA genes of fungi (18S, 5.8S and 28S genes) and differences within the spacer regions (ITS1 and ITS2) are the genetic basis for the organization of the fungi into taxonomic groups. After a detailed discussion of the principles that are known about the biological aspects of the ITS regions, the application of
using these spacer regions as molecular targets will be considered.

**rDNA complex**

The ITS regions are located in the rDNA gene complex of all eukaryotic cells between the 18S and 5.8S rDNA genes (ITS1) and the 5.8S and 28S rDNA genes (ITS2) (Fig. 1) [39]. The overall rDNA gene complex includes (from 5′ to 3′ orientation) the intergenic spacer region (IGS), which contains the external transcribed spacer (ETS) 1 region (also called the nontranscribed spacer) on the 5′ end and the ETS2 region on the 3′ end, along with a variable positioning of the 5S rDNA gene, the 18S rDNA gene, the ITS1 region, the 5.8S rDNA gene, the ITS2 region, and the 28S rDNA gene [40]. During early transcription in the cell nucleus, the initial precursor in the formation of RNA contains nucleotides for the 5′-ETS1/18S/ITS1/5.8S/ITS2/28S/ETS2-3′ subunit, referred to as the 35S to 45S rDNA transcription unit. This genomic unit, which contains the genes necessary for the formation of ribosomal RNA (rRNA), has been designated the rDNA gene complex of the fungal genome [41]. As the 5S rDNA gene is processed during transcription in a step distinct from the other rDNA gene products, it is not included as part of the precursor RNA transcript. Therefore, the 5S gene will be excluded from the discussion on the rDNA complex, although it is vitally important for the formation of ribosomes [42].

In the yeast *Saccharomyces cerevisiae*, the first eukaryotic cell to have its complete genome sequenced, the rDNA gene complex is roughly 9 kb in size and located on Chromosome XII [43]. This gene complex, which is repeated approximately 140 times on this chromosome, has a total repeat base count of approximately 1260 kb. As the size of the *S. cerevisiae* genome is around 13.4 megabases, the rDNA gene complex therefore comprises about 10% of the total genome. This multi-copied gene complex, containing both conserved and variable nucleotide sequence regions, serves as a reference point for evolutionary divergence studies because of its universal presence in all eukaryotic cells.

**rDNA complex genes**

All three genes within the rDNA complex have been used in studies on the molecular evaluation of fungi. The 18S-gene region is about 1800 bp in size with both conserved and variable domain sequences. Sequence variations within this region have been used to assess the taxonomic relationships of the major groups of living organisms and to separate genera and species based on sequence polymorphism [12]. However, the drawback to using this region for the identification of species is the relative sequence homology among fungal species and the need to sequence a large number of bases in order to do comparative analysis. The 5.8S region on the other hand is only about 160 bp long and highly conserved within major organism groups. Owing to this small size and conserved nature, it is not appropriate for phylogenetic studies to classify fungal species. However, this conserved sequence within major groups of microorganisms has been useful as an attachment site for universal primers to amplify flanking spacer regions within the eukaryotic genome [10]. Finally, the 28S-rDNA gene, which is around 3400 bp in size within fungi, also contains both conserved and variable nucleotide sequence regions. The variable domains of this large ribosomal subunit have also been used to allow comparisons from high taxonomic levels to the species level. In a database on large ribosomal subunit RNAs derived from eukaryotic cells, De Rijk et al. [44] reported that sequences within this 28S region ranged in length, from about 2900 bp in yeast to over 5000 bp in *Homo sapiens*. Much of the 28S-rDNA gene, however, is conserved among organism groups limiting the usefulness of this region for species identification [19,20].

**rDNA complex spacer regions**

Four transcribed spacer regions are located within the rDNA gene complex of fungi; two external to the 18S and 28S rDNA genes in the IGS noncoding region (ETS1 and ETS2) and two internal, flanking the 5.8S rDNA gene (ITS1 and ITS2) [39] (Fig. 2). Both ETS regions contain conserved sequences among fungal cells and although their biological role is not fully understood, both appear vital for early rRNA processing during transcription [45].

The ITS1 and ITS2 regions on the other hand, which flank the 5.8S rDNA gene, show extensive sequence diversity among major groups of eukaryotic microorganisms and even within species of the same organism group. Sequence sizes for each region vary from around 1000 bp in human cells to <300 bp in some yeast [46,47]. It was shown that these DNA spacer regions are also important for early transcription during rRNA processing.
Biological role of the ITS regions

The biological role of the ITS regions during processing of the rRNAs remains an intriguing puzzle, even though a number of functions have been suggested [41,45,47]. During the initiation of transcription, the primary 35S to 45S rRNA transcription unit is formed [41,48] (Fig. 3). This precursor transcript undergoes enzymatic removal of the four spacer sequences to become a primary rRNA transcript that is modified through RNA methylation and other base conversions and assembled into an 80S nucleolar ribonucleoprotein particle (RNP) [49,50]. The 80S RNP particle undergoes cleavage to form the 18S, 5.8S and 28S mature rRNAs ultimately combine with proteins to become functional ribosomes [51,52]. These functional ribosomes bind to mRNA and tRNA leading to protein production during translation [49].

Studies have shown that removal of one or both ITS regions prior to the initiation of transcription has a deleterious effect on the formation of mature rRNAs. Musters et al. [53] reported that removal of ITS1 prevented the formation of mature 18S rRNA and hence protein production during translation. Additionally, van der Sande et al. [54] showed that removal of the ITS2 sequence prevented the maturation of 5.8S and 28S rRNAs. Further work by Good et al. [55] confirmed the importance of the ITS2 sequence in the formation of functional ribosomes. They suggested that during RNA maturation, there appeared to be an interdependence in the role of the ITS region as a quality control mechanism to ensure that only functional rRNA was incorporated into the ribosome.

As DNA of ITS regions is removed and it is not part of the mature RNA molecule, they are considered non-coding regions of the genome. However, they are not normally identified as introns, but as intervening non-coding regions.
coding sequences or pseudo-introns because of their importance as precursors in protein manufacturing.

Applications
Identification methods

General approach
Various molecular techniques using the ITS regions as molecular targets for the identification of fungi have been evaluated. These methods include direct sequence analysis of amplified DNA, utilization of genus- or species-specific primers and oligonucleotide probes, and rDNA restriction fragment length polymorphism (RFLP) analysis. Most studies have shown that enough sequence variability exists among fungi within either the ITS1 or ITS2 region to allow for species identification. However, in rare instances the nucleotide sequence of one or both of the spacers is >99% similar between species (interspecies similarity), making it difficult to separate species by sequence information alone [56]. Additionally, strains within some single species have not shown enough variability (intraspecies variation), making it difficult to assign species based totally on sequence information because of this diversity [57,58]. This sequence difference among fungal strains and sequence similarity among species may in some cases represent a misidentification based solely on phenotypic characteristics or a mislabeling of the taxonomic positioning of the microorganism. Likewise, however, genotypic differences within the ITS regions of single species has also proven to be useful for typing [59]. Numerous examples will be discussed describing the reliability of using the ITS regions as molecular targets to identify and characterize fungi to the genus and species level.

Direct sequence analysis of amplified ITS DNA
The utilization of direct sequence analysis was an important technical development in the field of amplification-based genotypic pathogen identification [8]. The rDNA complex was shown to be a useful target because of the preservation of conserved gene sequences (the 18S, 5.8S and 28S rDNA) that flank highly variable signature sequences (ITS1 and ITS2 regions). Table 1 lists a number of studies [40,56,60–75] where direct sequence analysis of ITS regions was useful for the identification of both yeast and filamentous fungi.

The complete nucleotide sequence of the Candida albicans 5.8S rRNA coding gene and the flanking ITS regions was reported by Mercure et al. in 1993 [76]. Lott et al. [40] subsequently performed work using the ITS2 region of the rRNA gene to identify Candida species based on sequence variations. They evaluated multiple strains of C. albicans, as well as a single strain of C. parapsilosis. Their results showed that C. albicans displayed no intraspecies variation and that the ITS2 region sequences alone appeared species-specific to allow for the differentiation of C. albicans from C. parapsilosis. Botelho and Planta [67] later carried out sequence analysis of both the ITS1 and ITS2 regions of C. albicans and C. tropicalis. They found that both spacer regions contained sufficient sequence diversity to make either of them suitable as specific target sites for the identification of C. albicans. These researchers later developed two ITS2 oligonucleotide probes that showed promise for the identification of C. albicans. In another study, intraspecies sequence heterogeneity within Candida species was reported to be common in the ITS1 region with the ITS2 region showing stability for identification purposes [59].

Lott et al. [64] later determined the nucleotide sequences of the ITS2 region for 13 additional species from those reported earlier within the genus Candida. They showed that no two Candida species had identical sequences within the ITS2 region and that the base-pair sizes of this region varied fourfold among the yeast

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studies. To expand on this observation, Chen et al. [65] conducted a study to evaluate 34 yeast species from multiple genera for ITS2 sequence polymorphism. They also determined that enough variation existed within this region to develop a database composed of the ITS2 length and sequence polymorphism for these isolates. In this study, they also validated the database by testing over 400 clinical yeast isolates.

Pioneering work for the identification of filamentous fungi using the ITS region sequences was performed by Gaskell et al. [56]. They investigated ITS sequences within species of Alternaria (six species), Aspergillus (seven species), Cladosporium (four species), and Penicillium (eight species) to determine if enough sequence variations occurred that would allow for species identification. They discovered that the ITS regions of all four genera were distinct; however, they also showed that the ITS2 region alone was highly conserved among Aspergillus and Penicillium species. These workers suggested that the ITS1 region contained distinct regions that would allow for identification within these two genera. Henry et al. [69] subsequently showed, while testing Aspergillus species commonly associated with human disease, that the aspergilli could be identified from culture by sequence comparison of both the ITS1 and ITS2 regions. They applied this approach to clinical applications for the early identification of Aspergillus from culture. This method showed promise in enabling an earlier diagnosis to be made to help in the selection of the most effective antifungal agents to treat patients with invasive aspergillosis. This study also showed that intraspecies ITS sequence variations within the multiple strains of Aspergillus tested were minimal.

Following the studies on yeast by Lott et al. [40] and on filamentous fungi by Gaskell et al. [56], additional investigations were done using sequence analysis of the ITS regions to identify a broader range of fungi. Uijthof et al. [60] sequenced the ITS1 region of 33 strains of the Exophiala dermatitidis complex and two similar type species of Sarcinomyces phaeomuriformis (previously described as a granular form of E. dermatitidis) and Pullularia prototropha for comparison. Twenty of the E. dermatitidis complex strains had identical sequences within the ITS1 region when compared to a type strain of this species. All of these species had previously been identified as E. dermatitidis using phenotypic methods. Eleven additional strains, which differed by one to four nucleotide positions in the ITS1 region, were also identified as E. dermatitidis by phenotypic methods. The remaining two strains of the previously identified E. dermatitidis complex, however, had 30 and 31 bp differences when compared to the type strain of E. dermatitidis. They were subsequently identified as Exophiala species and E. jeanselmei var. heteromorpha, respectively. S. phaeomuriformis and P. prototropha also differed by 30 and 31 bases from the E. dermatitidis type strain, respectively. Uijthof et al. concluded in their study that it was possible to discriminate E. dermatitidis from other closely related black yeast using a signature sequence within the ITS1 region. This study illustrated the value of genotypic over phenotypic methods for identification. An oligonucleotide probe specific for E. dermatitidis was designed as a result of this study to discriminate this species from other closely related black yeast.

Sequencing of the ITS1 region has also proven useful for the differentiation of dermatophytic fungi included in the genera Epidermophyton, Microsporum and Trichophyton. This collection of fungi includes a heterogeneous group of more than 40 species, which are difficult at times to identify using phenotypic methods. Makimura et al. [61] determined the ITS1 sequences for six Trichophyton species which included two variants of T. mentagrophytes, four species of Microsporum and one strain of Epidermophyton floccosum. The size of the ITS1 region ranged from 175 to 293 bp, with sequence variability sufficient to identify the various species evaluated. To determine whether ITS1 sequence variations could be useful clinically to identify the dermatophytes, they also tested 11 clinical isolates of dermatophytes, which had been identified by morphological studies. Additionally, two strains of dermatophytes, which could not be identified due to the lack of typical microscopic structures, were also studied. ITS1 sequence analysis matched with the morphological identification in all 11 clinical isolates while the two phenotypically unidentifiable species were recognized as T. rubrum and T. violaceum by sequence comparisons.

El Fari et al. [77] recently showed the utility of using sequence comparison of the ribosomal ITS regions to identify T. tonsurans in an outbreak of tinea corporis in wrestlers. Forty-six isolates were evaluated, with 15 isolates unable to be identified by conventional culture methods. These isolates were all identified by ITS sequence comparisons that matched with the previous isolates, suggesting clonal reproduction of the population of this species.

Harmsen et al. [62] expanded the number of dermatophyte isolates sequenced to include eight species of Trichophyton, five species of Microsporum and three taxonomically closely related organisms, Chrysosporium keratinophilum, Cenomyces serratus and Gymnascus reessii. They also showed that the ITS1 region had enough nucleotide sequence variability to identify all these additional organisms.
In another analysis of dermatophytic fungi, Mochizuki et al. [78] applied ITS1 nucleotide sequence analysis to identify several clinical isolates that had atypical phenotypic results from previous testing. These organisms had been identified to species level using randomly amplified polymorphic DNA (RAPD) analysis and RFLP analysis of the mitochondrial DNA. It was shown that the identification by sequencing of the ITS region matched the identification made by the RFLP and RAPD profiles. Mochizuki et al. also reported that the sequence analysis showed greater reproducibility than either of the two other genotypic methods used.

Sequence analysis of the ITS regions for identification purposes has also been applied to other fungal pathogens where separation to individual species based on morphological characteristics is difficult. Sugita et al. [16,79] showed that the six medically relevant species of Trichosporon could be identified by sequence analysis within the ITS1 and ITS2 regions. They developed a database for Trichosporon species based on comparative sequence analysis of the ITS regions. Additionally, Makimura et al. [63] demonstrated the application of ITS1 rDNA sequence analysis for the identification of Malassezia species and Imai et al. [70] showed the utility of using the ITS region sequences for the identification of Paracoccidioides brasiliensis.

Finally, ITS sequence analysis was also applied to the identification of unusual fungal pathogens associated with invasive disease. Kuhls et al. [73] applied sequence analysis using both the ITS1 and ITS2 regions to identify Trichoderma longibrachiatum and T. citrinoviride which had been isolated from two rare cases of human disease. Iwen et al. [72] showed the advantage of using the ITS regions for the recognition of the fungus Gymnascella hyalinospora, an unusual mould isolated from a case of systemic disease.

**Genus/species-specific primers and oligonucleotide probes**

Once it was demonstrated that ITS sequences could be used for the identification of fungal species, additional studies focused on the recognition of ITS signature sequences for the development of specific primers and probes. Most of the work to date has been in the creation of probes to identify Candida species. Fujita et al. [80] constructed probes from the ITS2 region for the identification of C. albicans, C. tropicalis, C. parapsilosis, C. krusei, and C. glabrata. They used a micro-titer plate enzyme immunoassay (EIA) with a digoxigenin-labeled probe method and showed that probes designed from this spacer region were useful for the identification of these Candida species. They also showed that this EIA assay was highly sensitive and was able to detect amplified DNA from as few as two C. albicans cells per 0.2 ml of blood. To expand on this highly sensitive methodology, Martin et al. [81] recently developed a reverse-hybridization line probe assay. This assay, combined with PCR amplification of the ITS region, detected and identified a number of clinically significant fungal species, with a detection limit of 2–10 cells ml⁻¹.

The EIA approach developed by Fujita et al. [80] was later applied to the identification of Candida species in blood cultures [82]. The combined PCR and EIA method used in this study correctly identified five candidal species detected from 73 blood culture bottles that had been verified by culture. This work has been expanded for the rapid identification of C. albicans, C. parapsilosis and C. tropicalis in a single reaction tube by a 5'-endonuclease assay using fluorescent-labeled DNA probes that bound to signature sequences within the ITS2 region [83]. These probes detected and correctly identified all three species in 58/61 (95.1%) positive blood cultures with no false-positive results.

Elie et al. [84] constructed probes from an additional 13 Candida species pathogenic for humans. These species included C. guilliermondii, C. kefyr, C. lambica, C. lusitaniae, C. pelliculosa, C. rugosa, C. zeylanoides, C. haemulonti, C. norvegica, C. norvegensis, C. utilis, C. viswanathii and C. dubliniensis. These probes along with the five previous probes designed by Fujita et al. [80] were tested against all 18 Candida species, as well as 17 other species and strains of fungi. Sixteen of the 18 probes were specific, with some cross-reaction occurring between C. guilliermondii/C. zeylanoides and C. glabrata/S. cerevisiae. These workers concluded that the probes designed from the ITS2 region were reliable for the molecular identification of Candida species.

To expand on the use of specific probes to identify Candida species, a new technology using molecular beacons has also been developed. Molecular beacons are small nucleic acid hairpin probes that brightly fluoresce when bound to their targets. They have significant advantage over conventional nucleic acid probes because they exhibit a higher degree of specificity with better signal-to-noise ratios [85,86]. Park et al. [87] used a species-specific molecular beacon developed from the ITS2 region for the rapid identification of C. dubliniensis. By using this hairpin probe, these researchers were able to identify 100% of 23 C. albicans and C. dubliniensis strains after PCR amplification. This work suggested that molecular beacons may be promising new probes for the rapid detection of Candida species.

Species-specific oligonucleotide primers that amplify a specific portion of the ITS region have also been designed and tested. Both Sugita et al. [88] and Motoyama et al. [89] used this approach to identify...
Trichosporon asahii and P. brasiliensis from culture. They each designed primers for a target region within the ITS2 area that was specific for the fungus tested with positive amplification indicative of the presence of the fungus.

Larena et al. [90] also designed an oligonucleotide primer that was specific to amplify DNA from fungal species that had been classified as Ascomycetes by observance of characteristic sexual reproductive structures. Their assay proved useful in the amplification of these species and they suggested that this method could also be used to determine affinities of asexual state for fungi with their perfect states by similarity in sequences within the ITS region. The utilization of species-specific primers in amplification assays for the identification of fungal pathogens appears likely to be evaluated further in future studies.

rDNA restriction fragment length polymorphism analysis
Amplified DNA can be cleaved with a restriction endonuclease and evaluated electrophoretically in a process referred to as post-amplification restriction fragment length polymorphism (PCR–RFLP) analysis [91]. Variations in the number and size of the restriction fragments have been used for the identification of microorganisms. The PCR–RFLP analyses when applied to amplified products of the 5.8S ribosomal DNA-intervening ITS regions has shown promise in distinguishing between different fungal species. In combination with morphological analysis, the results of PCR–RFLP have been useful for the characterization of isolates in both clinical and commercial applications [91–94]. Described in the following section are some of the clinical applications of this technology using the ITS region targets.

PCR–RFLP analysis of fungal DNA from the amplified ITS region sequences was originally done on species within the black mould group. This was carried out because of the difficulty in identification of these moulds by morphological methods. Uijthof & de Hoog [95], performed PCR–RFLP analysis on 22 type strains of black moulds in the genera Exophiala and Phaeococcymyes. They used the restriction enzymes DdeI, HaeIII, HhaI, HinII, NdeII, MsPI, Rsal, TaqI and AluI in their analysis. When comparing the restricted fragment profiles provided by these enzymes, they showed that species within Exophiala could be distinguished from one another following PCR–RFLP of the rDNA ITS amplicon, depending on which enzyme was used. They suggested that subsequent studies on more strains of each species be conducted in order to establish intra-species variability, as this was not evaluated.

Attili et al. [66] analyzed restriction profiles from 13 strains of Fonsecaea pedrosi and three strains of F. compacta. Both species are known to cause the disease chromoblastomycosis, which is characterized by the presence of cauliflower-like lesions emerging from the skin in people living in the tropical and subtropical regions of the world. Debate over the years has centered on whether these two species should be combined into one. An approximate 650-bp amplicon, which contained the ITS1–5.8S rDNA–ITS2 region, was evaluated for all strains against seven restriction enzymes. Identical patterns among strains and between the two species were generated with HinII, HaeIII and Rsal, with minor restriction fragment variations observed between species using the enzymes MsPI, HhaI, DdeI and TaqI. The minor differences found in restriction patterns in this study did not appear to correspond to the original morphological identification. Additionally, the RFLP patterns of the ITS amplified region for F. pedrosi and F. compacta generated using HinII, DdeI and HaeIII were indistinguishable from those known for Exophiala species [66]. This finding was not unexpected, however, because of the close relationship of Exophiala and Fonsecaea within the Ascomycete family [96]. Additionally, even though Attili et al. [66] showed that restriction fragment patterns were very similar between F. pedrosi and F. compacta no matter which enzyme was used, they also showed, following sequence analysis, that ITS nucleotide sequences varied extensively between these two species. Their results showed the limitation of PCR–RFLP in the evaluation of this group of fungi. The debate of whether F. pedrosi and F. compacta should be combined into one species will continue. Results from this study suggested that the high variability in the ITS sequences between these species made it impractical to combine them and that a renewed taxonomic study of Fonsecaea is overdue.

Further work with the black fungi using PCR–RFLP analysis of ITS regions was done by Ahmed et al. [97] for the identification of Madurella mycetomatis, an agent significant for causing mycetoma. The genetic variability of the ITS regions of 25 different clinical isolates of M. mycetomatis, along with closely related strains of M. grisea, Pyrenochaeta mackinnonii, P. romeri, P. unguis-hominis and Chaetosphaeronema larense were evaluated following digestion with CfoI, HaeIII, MsPI, Sau3A, Rsal, and SpeI restriction enzymes. The RFLP patterns generated by these six restriction enzymes produced banding profiles that were in full agreement with the nucleotide sequences of the ITS regions for the M. mycetomatis strains. Furthermore, the other five species tested, which were all closely related by phenotypic characteristics, could be clearly differentiated from M.
mycetomatis by their RFLP patterns. These results appeared to be in conflict with earlier results by de Hoog et al. [98] who showed that agents of mycetoma of the same species were highly diverse in their restriction pattern. These researchers suggested that testing of geographically diverse strains of M. mycetomatis might show restriction pattern differences. In a recent study by Caligiore et al. [99], 12 isolates of dematiaceous agents of chromoblastomycosis and phaeohyphomycosis were evaluated by PCR–RFLP analysis of the rDNA region. These results showed that a high degree of inter- and intraspecies variability did exist. This study included fungal species that had been evaluated previously by Ahmed et al. [97] and de Hoog et al. [98].

Early work to assess yeast using the ITS region as the target in PCR–RFLP analysis was performed by Messner & Prillinger [100] to differentiate 10 genotypically distinct Saccharomyces species. They amplified the complete ribosomal repeat in two parts, the 18S rDNA, which included both ITS regions and the 5.8S rDNA area (total length of 2900 bp), and separately the 25S rDNA (total length of 3300 bp). Restriction fragments generated from these two PCR products by nine restriction endonuclease enzymes yielded characteristic patterns by which unknown isolates of Saccharomyces could be assigned to species. McCullough et al. [101] simplified this methodology by decreasing the size of the PCR amplicon, using an approximately 650–850-bp region of the genome which included the ITS1 and ITS2 regions. They showed that the ITS–PCR ribotype pattern obtained with HaeIII and MaeI could separate all species of Saccharomyces species with the exception of S. bayanus and S. pastorianus. These results confirmed the findings of Messner & Prillinger [100] while simplifying the methodology by markedly decreasing the size of the PCR amplicon required to successfully differentiate species of Saccharomyces. Further work by Montrocher et al. [102] using RFLP analysis of the PCR-amplified rDNA ITS region, helped to clarify the taxonomic position of species within the Saccharomyces genus.

A PCR–RFLP based identification system, using the ITS region was also developed for the identification of Malassezia species by Gupta et al. [103]. Following amplification of the 28S-rDNA gene and ITS regions with digestion by EcoRI, NcoI, and AvaI restriction enzymes, five of the seven Malassezia species were readily distinguishable by the RFLP patterns generated. It was, however, difficult to differentiate M. furfur from M. pachydermatis and M. globosa from M. restricta using this method. Additionally, intraspecies variations were noted within M. furfur, M. sympodialis and M. slooffiae. These researchers suggested that even though variations were within species, the PCR–RFLP procedure may prove to be the preferred analysis to distinguish among Malassezia species. They also stated that other regions of the genome, including protein-encoding genes, may be a better target and provide further insight into intraspecies variations and thus are useful in epidemiological studies. Additional studies using PCR–RFLP analysis of the rDNA ITS region were performed by Granchi et al. [104] on 30 species of yeast of oenological significance. They showed that using DraI or HaeIII restriction enzymes allowed for a more precise identification than traditional biochemical methods.

Identification of Candida species using RFLP analysis of the rDNA repeat has also been achieved [105,106]. Williams et al. [106] evaluated eight species of Candida by PCR–RFLP analysis of the ITS regions. In this study, the enzyme BflI provided for the greatest level of species discrimination. They applied their genotypic method to the identification of Candida species from a range of sources and felt that this was a reliable method to differentiate Candida species.

PCR–RFLP analysis of the rDNA repeat was also used for the molecular differentiation of the dermatophytic fungal species. Jackson et al. [107] amplified the contiguous ITS and 5.8S rDNA regions of 17 common dermatophyte species and digested the amplified ITS products with the restriction endonuclease MvaI. This process produced unique and easily identifiable fragment patterns for a majority of dermatophytes. However, closely related taxon pairs T. rubrum/T. soudanense and T. quinckeum/T. schoenleini could not be distinguished using this methodology.

As stated, the utilization of restriction profiles has been useful for the identification of some fungi. Additionally, this methodology has also shown promise for the typing of fungal pathogens for epidemiology purposes. Typing of fungi will be discussed further in the section on utilization of the ITS regions for epidemiological investigation.

Phylogenetic studies

Introduction and general approach

Fungal systematics or taxonomy includes the areas of phylogenetic analysis, nomenclature, and species identification. An important step to name and identify fungal pathogens is to align them into groups of organisms with common characteristics. This aim of classification is based on the development of phylogenetic trees to trace back the most probable course of evolution between groups. Numerous phenotypic methods have been used to establish these relationships; however, molecular methods have become the mainstay of phylogenetic
studies [18]. These molecular methods include determination of the genomic G+C ratio, DNA–DNA hybridization, comparative sequence analysis, and comparisons using electrophoretic approaches such as RFLP analysis. The frequent genetic targets for phylogenetic studies are usually the genes that code for rRNAs in the rDNA gene complex [108]. These genes include the highly conserved 5.8S, 18S and 28S rDNA genes and the alternating variable regions or divergent domains (ITS and the IGS regions). Comparative nucleotide sequence analysis using the conserved 18S and the 28S-rDNA gene regions have been used to distinguish between different genera [13,31,96,109–115]. The 5.8S rDNA gene on the other hand has not been useful in assessing the phylogenetic relationships as the sequence is generally too small and has little variability within major groups of organisms. As the noncoding ITS region is highly polymorphic, Jorgensen & Cluster [116] predicted that this variability would prove useful in resolving relationships between closely related taxonomic relatives. White et al. [10] subsequently proposed the use of universal fungal primers based on highly conserved regions of the rDNA for PCR amplification of the ITS1–5.8S–ITS2 rDNA region followed by sequence comparison. Table 2 illustrates some of these studies [40,54,57,60–62,64,66,73,74,79,84–86,88,95,102,103,111,114,117–138] that have used sequence variations within the ITS regions to classify fungal species. Examples using the ITS regions as tools for phylogenetic analysis of fungi are described. For a detailed review on fungal taxonomics, refer to Guarro et al. [18].

Candida species

*C. albicans* is the most common cause of invasive fungal disease; however, several other closely related species have emerged as opportunistic pathogens in immunocompromised patients [139]. The classification of *Candida* species has typically been based on phenotypic characteristics; however, among some species phenotypic differences are minimal making it difficult for differentiation. For example, Sullivan et al. [140] described a new species of *Candida* as *C. dubliniensis*, where common phenotypic characteristics for identification were indistinguishable from *C. albicans*. It is important to recognize this species, as it has a propensity to develop resistance to fluconazole, an antifungal drug commonly used to treat candidiasis [141]. The original identification and phylogenetic positioning of *C. dubliniensis* was based in part on genomic methods, which included DNA fingerprinting and phylogenetic analysis of the rDNA gene sequences including the ITS region [140]. Following the recognition of this species, phenotypic markers have now been described which appear useful in differentiating *C. dubliniensis* from other yeast species [142,143].

Nucleotide sequences for the DNA encoding the ITS1 and ITS2 regions have also been determined for numerous other species of *Candida* [40]. Recently, Elie et al. [84] evaluated seven phenotypically confirmed strains of *C. famata* by sequence analysis of the ITS2 region. They showed that major sequence differences were observed in the spacer region among these strains, suggesting that this group of isolates may in fact contain multiple species. These studies revealed that additional molecular characterization of the *Candida* species is needed for further taxonomic resolution within the genus.

**Dermatophytes**

The dermatophytes—fungi capable of invading keratinized tissues to produce ringworm—include over 40 species [144]. Techniques utilizing the G+C content of chromosomal DNA [145], total DNA homology [146], RFLP analysis of mitochondrial DNA [147,148], and base sequence comparison within the 18S [126] or 28S rDNA genes [113] have been done. Comparative sequence analysis of the 18S and 28S rDNA gene regions showed similarity among the dermatophytes suggesting that they probably originated from a common ancestor. As the nucleotide sequences within these regions were conserved among the dermatophyte species could not easily be delineated. Additionally, the advent of mole-
cular biological techniques has called into question the organization of species and subspecies within these genera. The evaluation of the more divergent spacer regions within the genome of the dermatophytes has therefore been undertaken to help clarify the classification of this group.

Phylogenetic analysis using the ITS regions was initially done using only the ITS1 region of the rDNA gene as a marker. Makimura et al. [125] evaluated 26 strains of the *T. mentagrophytes* complex and 11 strains of *T. rubrum* from both animal and human origin. They concluded that it was possible to differentiate these species using sequence comparison within the ITS1 region and to demonstrate a phylogenetic relationship between these two species. In a follow-up study, Makimura et al. [61] evaluated additional strains and species from all three dermatophyte genera. They again showed that it was possible to phylogenetically classify these organisms by using the base-pair sequences within the ITS1 region for comparison. Gräser et al. [123] subsequently studied both the ITS1 and ITS2 region sequences of 53 strains representing 38 species and varieties of the dermatophytes. They concluded that the phylogeny of this group broadly reflected its ecology, that is that major groupings could be distinguished within the geophilic, zoophilic or anthropophilic species.

In a follow-up study, they expanded their evaluation by testing strains representing 27 species and varietal names (many of them invalid or long considered synonyms of recognized species) within the *T. mentagrophytes* and *T. tonsurans* complexes along with taxa. Multiple molecular methods [124] were used. These methods included sequencing of the ITS1 and ITS2 regions, PCR fingerprinting and amplified fragment length polymorphism analysis. They showed that 24 of the taxon names studied corresponded to only five phylogenetic species, *T. mentagrophytes*, *T. tonsurans*, *T. interdigitale*, *T. simii* and *T. erinacei*. Their results, however, in some cases conflicted with current taxonomy based on morphology and physiological characteristics, again showing the unreliability of purely phenotypic species delimitation.

Gräser et al. [149] further evaluated the genus *Trichophyton* to determine the taxonomic position of species closely related to *T. rubrum* by applying several molecular methods, which included ITS sequencing. They reported that two clades could be distinguished on ITS data alone. The clinical picture within each clade showed a clear difference, with clade 1 species primarily causing tinea capitis as an endothrix infection of the hair and clade 2 species predominately causing tinea pedis, onychomycosis, and tinea corporis. They developed a bootstrap consensus tree for all 17 species evaluated.

Recently, Summerbell et al. [127] sequenced the ribosomal area that spanned the two ITS regions and the 5.8S rDNA region of asexual, anthropophilic dermatophyte species. These isolates were morphologically similar to *T. rubrum* and to members of related clades in the *T. mentagrophytes* complex. They confirmed that ecologically and phenotypically separated species have only small differences in the base-pair sequence within the ITS regions. Owing to this small difference, these researchers recommended that additional genomic loci be analyzed in the evaluation of species status within this group of fungi. Jackson et al. [107] using PCR–RFLP analysis on amplified ITS products also showed it was difficult to separate all related species within *Trichophyton* using ITS–PCR.

The validity of taxa of the *Microsporum canis* complex was studied by Gräser et al. [150] using results of sequencing of the ITS regions. They evaluated several previously identified species. Based on sequence results, they suggested that the isolates be classified as three species, *M. canis*, *M. ferrugineum* and *M. audouinii*.

**Penicillium marneffei**

*P. marneffei*, a well-recognized human pathogen which is considered the third most common cause of disseminated infection in patients with AIDS in parts of southeast Asia, has recently undergone a change in taxonomical position based on an evaluation of the ITS sequences [151]. Segretain [152] gave the first mycological description of *P. marneffei* and classified this fungus in the section *Asymmetrica* subsection *Divaricata* of Raper & Thom's [153] taxonomic treatment of *Penicillium* species. This section was equivalent to Pitt's [154] *Penicillium* subgenus *Furcatum*. Subsequently, however, *P. marneffei* was assigned to the subgenus *Biverticillium* rather than the subgenus *Furcatum* because of phenotypic characteristics more closely related to the former subgenus [154]. LoBuglio & Taylor [117], using the nucleotide sequence within the ITS regions and the 5.8S rDNA gene, confirmed that the phylogenetic position of *P. marneffei* was more related to species of *Penicillium* subgenus *Biverticillium*. Knowledge of the phylogenetic position of *P. marneffei* has allowed these researchers to design unique oligonucleotide primers that target the nuclear rDNA ITS region to specifically amplify this fungus in molecular assays.

**Trichosporon species**

*Trichosporon* species are associated with a variety of infections including deep-seated mycoses, mucosal infections and superficial infections. Recently, these species have emerged as major causes of opportunistic infections in immunocompromised patients [155,156].
Over the last decade, genetic methods have significantly changed the taxonomy of *Trichosporon* [157,158]. Sugita *et al.* [68,135] recently described a phylogenetic tree for the *Trichosporon* species constructed from a comparison of nucleotide base sequences in the 18S and 28S rDNA. However, owing to similarities in the nucleotide sequences within these rDNA regions, it was difficult to identify *Trichosporon* to the species level. In a further attempt to classify all species within this genus, Sugita *et al.* [79] sequenced and analyzed both the ITS1 and ITS2 regions of 17 species and five varieties of *Trichosporon*. Even though it was difficult to distinguish among some of the species because of >99% sequence similarities within the ITS regions, they still were able to construct phylogenetic trees for species based on comparative sequence analysis. Based on this information, they concluded that six medically relevant species, *T*. *asahii*, *T*. *asteroides*, *T*. *cutaneum*, *T*. *inkin*, *T*. *mucoides* and *T*. *ovoides*, could be readily identified by their ITS sequences. The ability to recognize individual species of *Trichosporon* appears important, since the severity of disease caused by these pathogens may be species associated [159].

**Malassezia species**

*Malassezia* species are lipophilic yeasts usually associated with causing a superficial skin infection known as pityriasis (tinea) versicolor; however, they can also cause a disseminated infection in immunocompromised premature infants referred to as catheter-associated *Malassezia* sepsis. The diversity of species within this genus has not been fully understood and with the development of molecular techniques, new species within the genus have been discovered. Guillot & Guého [112] sequenced portions of the 28S-rDNA gene as a method to delineate among *Malassezia* species. They showed that species identification following sequence analysis of this region matched well with morphological and serological characteristics documented by other authors. Guého *et al.* [160] followed this report with studies on morphological characteristics, physiological features, and ultrastructural studies to identify additional species within the genus. As direct sequencing of the 28S rDNA gene was time-intensive because of the relatively large sequence needed for differentiation of species, Gupta *et al.* [103] attempted to identify species using only a portion of the 28S rDNA gene and both ITS regions as a molecular target. Using RFLP analysis of the PCR product generated, they were able to delineate *M*. *furfur*, *M*. *symposialis* and *M*. *slooffiae*, which are all physiologically similar. *M*. *obtusa*, another species which is similar to *M*. *globosa* and *M*. *restricta*, could also be differentiated using this approach. However, *M*. *globosa* and *M*. *restricta*, which were readily distinguishable by differences in cell morphology, were difficult to separate on the basis of the molecular characters studied. Intraspies variation within the ITS region in some of the species of *Malassezia* were also noted. Additional studies were suggested to evaluate further the species characteristics within *Malassezia*.

**Direct amplification from clinical specimens**

**Introduction**

Early recognition of an invasive fungal infection is difficult, with primary diagnosis frequently made at autopsy [161]. The rapid and accurate diagnosis of an invasive fungal infection is important since early antifungal treatment is associated with improved outcome [162–165]. Additionally, the recent introduction of new antifungal agents with differing spectra of activity has led to the need to develop treatment strategies predicted on an accurate species identification [166–169].

Several molecular methods have been evaluated for obtaining a diagnosis directly from clinical material using a variety of specimens and molecular targets [170]. From the rDNA gene complex, the 18S-rDNA gene has been the major target for the detection of *Candida* and *Aspergillus* from blood and bronchoalveolar lavage (BAL) fluid [12,171–173]. Melchers *et al.* [174] tested BAL samples from a small group of neutropenic and nonimmunocompromised patients for the presence of *Aspergillus* DNA using this 18S rDNA target. They showed that it was possible to detect the aspergilli in these samples and thereby to identify neutropenic patients at risk for invasive aspergillosis (IA), even though a positive result did not always correlate with invasive disease. Subsequent work by Verweij *et al.* [175] using the same 18S rDNA target and also testing BAL fluid, showed similar results for utilization of this molecular technique to aid in the diagnosis of IA in neutropenic patients. Einsele *et al.* [12] later used the 18S rDNA target to test blood for the presence of *Candida* and *Aspergillus* DNA. Their pilot study showed promise for the detection of fungal pathogens directly from blood, with increased sensitivity accomplished by testing multiple samples on individual patients. All of these studies using the 18S rDNA gene target showed promise for detection of fungal DNA, but frequently allowed for the identification of fungal genera only because of limitations due to sequence similarity within this region.

**Sterile body sites**

Recent studies using ITS regions as molecular targets have reported the detection of fungal pathogens from sterile body sites such as blood and serum [176–178]. In...
experimentally infected rabbits, Bougnoux et al. [176] performed a comparative analysis between serum and whole blood samples for the diagnosis of invasive candidiasis (IC) using nested PCR with primer pairs that amplified specific ITS targeted areas. They were able to identify Candida species within the blood and serum of infected rabbits in most of the samples tested. Their results also showed that serum was slightly more reliable than whole blood for the diagnosis of IC, suggesting that PCR inhibitors in the blood, such as hemoglobin, may have contributed to this difference.

Differences in sensitivity between blood and serum were further addressed by Loeffler et al. [173] while comparing human whole blood with plasma and detecting Aspergillus DNA. They showed that PCR performed on whole blood was more sensitive for the detection of aspergilli than plasma, in contradiction to those results reported by Bougnoux et al. [176]. They suggested that extraction of DNA from plasma appeared to be more complex and that inhibitors in the plasma such as albumin and globulins might be present in the extracted DNA. They recommended that an extraction procedure that performs a complete protein precipitation, such as the labor-intensive technique associated with the phenyl-chloroform-isamyl alcohol procedure, be used in order to remove these plasma inhibitors.

Another direct procedure was performed using an experimental mouse model of fusarium infection [177]. In this study, a primer pair that amplified a portion of the 5-8S and 28S-rDNA gene containing the ITS2 region of Fusarium spp. was used to detect Fusarium DNA in both tissues and in Fusarium-spiked human blood. Their results demonstrated a high degree of sensitivity and specificity for the detection of Fusarium in both the tissues from the infected mice and spiked human blood using the ITS2 region as a target.

In other experiments testing human samples, Wahyuningisih et al. [178] showed it was possible to detect C. albicans DNA in sera from patients with invasive candidiasis (IC). They used a PCR amplification assay followed by species identification with a C. albicans-specific hybridization probe of the ITS2 region. In this study, all negative control patients were PCR negative and all patients with culture recognized IC tested positive by PCR. These researchers showed that the ITS–PCR assay was simple, specific, and more sensitive than routine blood culture to detect candidemia. They suggested that the molecular detection of C. albicans in serum was valuable in the identification of patients at risk for IC.

**Dermatological specimens**
The accurate identification of a dermatophyte species by culture is necessary because of the growing number of antifungal agents with differing spectra of activities to treat these various fungi. The conventional culture and biochemical methods for identification of dermatophytes are time consuming and may be rendered difficult by a paucity of morphological and biochemical features in some isolates. Owing to this difficulty in identification, genotypic methods for the direct detection and identification of dermatophytes in tissue have been initiated.

Some of the original work to identify dermatophytes directly in clinical specimens using ITS–PCR was done by Fari et al. [179]. They used a primer set that amplified both the ITS1 and ITS2 regions of T. rubrum followed by a T. rubrum-specific hybridization probe derived from the ITS2 region of the rDNA operon. They applied this assay to nail samples from 35 patients with confirmed T. rubrum-caused onychomycosis. In this assay, they failed to detect T. rubrum in 25 of the samples. These researchers felt that the DNA extraction method used may not have been effective for preparing fungal DNA from nail material. In a subsequent evaluation of two T. rubrum-positive skin samples using the same assay and DNA extraction method, they were able to amplify the fungal DNA. This follow-up testing showed their PCR/hybridization method had potential for the rapid detection of dermatophytes in clinical material.

Turin et al. [180] also evaluated dermatological clinical specimens using PCR methodology with multiple primer pairs that amplified fragments of the highly conserved gene coding for the 18S rDNA and the adjacent ITS spacer regions. The primer pair specifically amplified the ITS1 and ITS2 regions and allowed for the detection of dermatophytes in 10/12 positive samples with no false-positive results. Nail samples were not distinguished from skin or hair samples in this study and no mention was made regarding the previously observed problem with nail samples. Turin et al. concluded that this assay could be used for early recognition of fungal pathogens in clinical samples, as an alternative tool to conventional detection methods.

**BAL specimens**
Bronchoscopy and the collection of BAL samples offer a reliable means for obtaining specimens from deep within the respiratory tract for the detection of pathogenic microorganisms. However, the detection of Aspergillus species from BAL fluid by culture in high-risk patients lacks sensitivity, as it is negative in approximately 50% of patients with proven invasive pulmonary aspergillosis (IPA) [172]. Furthermore, as Aspergillus both colonizes and contaminates the respiratory tract, a positive culture may not indicate infection, making interpretation of a positive result difficult.
To increase the sensitivity and specificity of BAL fluid samples for the diagnosis of fungal infections, PCR methodology has been evaluated in a number of studies. Pioneering work in this area was done by Melcher et al. [174] to detect *Aspergillus* species using the 18S-rDNA gene as the molecular target. This study, along with work by other researchers using this targeted region, showed the potential for using a PCR-based assay in the diagnosis of IPA in high-risk patients [172,181]. Einsele et al. [181] recently showed there was a significant association between BAL that tested positive for *Aspergillus* DNA by PCR and the development of IPA, when patients were colonized prior to intensive immunosuppressive therapy. They suggested that these patients would benefit from systemic antifungal prophylaxis.

All of the early work for the detection of *Aspergillus* DNA in BAL fluid was done using the 18S-rDNA gene as a target. Most studies using the ITS regions as targets were conducted on BAL samples taken for the detection of *Pneumocystis carinii*. Tang et al. [182] using a single-tube-nested PCR showed that this technique had sufficient sensitivity to detect *P. carinii* in BAL fluid from 10 patients with *P. carinii* pneumonia (PCP), whereas all 10 BAL specimens from patients with other diseases were negative by PCR. Subsequent work by Keely and Stringer [183] showed that changes in the ITS sequence among *P. carinii* strains detected in BAL fluid during different episodes of PCP, supported the hypothesis that recurrent disease was caused by re-infection with a new *P. carinii* strain rather than by reactivation of latent organisms. This work was also later validated by Tang et al. [184] who showed that two types of *P. carinii* ITS sequences derived from a single BAL specimen, indicated that the patient was infected by two different strains of *P. carinii*. Multiple genotypes in a single BAL specimen were also seen by Hosoya et al. [185]. In a review of the current literature, we were unable to identify studies testing BAL fluid specimens for the presence of either *Aspergillus* or *Candida* species DNA using the ITS regions as targets.

**Deep tissue**

The diagnosis of an invasive fungal infection with species confirmation usually requires both histopathological evidence of the fungus in deep tissue obtained by biopsy and culture of the fungal pathogen from this clinical material [162,164]. Isolation of a mould or yeast from deep-tissue samples that contain focal lesions, however, is frequently not accomplished [186]. Additionally, culture of tissue is not always performed such as that which occurs at autopsy, where the invasive infection was not suspected and became an incidental finding. At postmortem examination, tissue is normally placed into a formaldehyde fixative for histopathological examination thus making it unsuitable for culture. In cases where an invasive fungal disease was detected by histology and no culture was performed a definitive identification of the pathogen is not possible. However, molecular diagnostic tests to evaluate fungal DNA in archived tissue has been shown to be valuable in some cases.

Only limited success has been achieved in the testing of fixed tissue for the presence of fungal DNA. For example, in situ hybridization tests to detect fungal-specific DNA have been attempted but lack sensitivity and are incapable of detecting specific sequences in degraded DNA found in formalin-fixed tissue [187–190]. Likewise, a number of other investigations have also shown the difficulty in performing PCR amplification on the DNA extracted from formalin-fixed tissues with or without paraffin embedding [191–193].

Karlsen et al. [194] evaluated in detail the effects of fixation of tissue and the effect on PCR amplification. Their results showed that DNA in tissues fixed for longer than 215 h could not be amplified if the PCR product was more than 200 bp, unless an excessive number (50–80) of PCR cycles were used. They showed that formaldehyde neither fragmented nor reduced the quantity of DNA, but changed the structure of DNA. They suggested that the double-stranded helical structure of DNA was transformed into single-strand DNA through a slow and inevitable process and that formaldehyde reacted with the bases in DNA causing a hydroxymethylation, which hindered primer annealing.

A further evaluation of methodology to amplify fungal DNA from tissue preserved in formaldehyde using PCR was done by Wildfeuer et al. [195]. They performed a nested PCR assay targeting the gene for cytochrome P450L1A1 using as a comparison both fresh and formalin-fixed homogenized tissue from *C. albicans*-infected mice. Their results showed that the sensitivity of the PCR assay was reduced by a factor of more than 1000 when the tissue was fixed in formalin for over 7 days. Douglas & Rogers [196] also attempted to amplify fungal DNA from fixed tissue using a 250-bp region target near the 3′ end of the 18S rDNA gene. In multiple attempts, they too failed to amplify DNA in the fixed samples. Successful amplification of fungal DNA in formalin-fixed tissue has, however, been reported. Kanda et al. [197] extracted DNA from formalin-fixed paraffin-embedded material of mitral valve and lung tissue of a patient with suspected IA. They used an *Aspergillus*-specific primer set and were able to amplify a 687-bp fragment within the 18S rDNA gene. In addition to this study, other investigators had promising results in the amplification of DNA from formalin-fixed paraffin-
embedded tissue to detect other eukaryotic microorganisms [198]. All of these previous studies had utilized nonspacer region rDNA complex targets. However, one study utilized the ITS sequence regions as targets on parafin-embedded tissue by PCR amplification. Lee et al. [199] amplified *P. carinii* DNA from fixed and embedded tissue sections using a method described by Greer et al. [200]. In this study, a variety of *P. carinii* strains were typed by a comparison of sequence variations. It is unknown in this study how long the tissues had remained in fixative, since they had been obtained from a variety of sources. Studies utilizing amplification testing of fixed tissue suggested that the limiting factors in detecting DNA included the size of PCR product (<200 bp), the number of PCR cycles, the fixation time (<8 h) and a nested PCR method.

As amplification of DNA from fixed tissue was problematic, assays were subsequently designed to detect fungal DNA from fresh tissue. Tsolaki et al. [201] amplified *P. carinii* DNA from archived lung tissue taken at postmortem exam and frozen at −70 °C. In this study, they evaluated the genetic similarity from pre-AIDS era isolates of *P. carinii* and contemporary isolate spacer regions. Hendolin *et al.* [186], using a PCR-based method with the ITS regions as targets, were able to detect fungal pathogens in fresh tissue. They used tissue from patients with suspected or proven deep-seated fungal infections and showed that the ITS–PCR procedure provided for the rapid detection and identification of fungal pathogens in tissue. They also showed that this procedure had increased sensitivity over both direct microscopy and culture.

**Environment**

Routine culture detection of fungal pathogens in environmental samples is an expensive and slow process, which frequently leads to false-negative results because of the low sensitivity of the culture method. Molecular methods using the ITS regions as targets in enzymatic amplification assays have been shown to be reliable for the direct detection of the true pathogen *Histoplasma capsulatum* var. *capsulatum* (teleomorph = *Ajellomyces capsulatus*) in environmental samples. An ITS–PCR method has been developed to detect *H. capsulatum* in soil suspected to be contaminated with this fungus [202]. A two-stage PCR protocol employing both fungal-specific primers and nested primers specific for the ITS region of *H. capsulatum* was used. It was estimated that the level of detection was 10 conidia, and that soil could be analyzed for the presence of *H. capsulatum* in less than 2 days.

In other environmental testing, Bartlett *et al.* [203] evaluated air samples for the presence of *P. carinii* using ITS region-specific primers. Their aim was to evaluate the epidemiology of *P. carinii* to gain more knowledge on where the organism entered into the environment and how it was transmitted to humans. They extracted DNA from filters exposed to sampled air from numerous locations including *P. carinii*-infected patient homes and hospital rooms, non-*P. carinii*-patient hospital rooms and from other negative control rooms. They detected *P. carinii* in 57% of the *P. carinii*-infected patient rooms and in 29% of hospital rooms that did not house infected patients, but were unable to detect *P. carinii* in any of the control rooms.

These studies illustrated the usefulness of molecular assays to detect fungal pathogens from the environment. The investigation evaluating *P. carinii* in the air also showed that it was possible to perform extensive molecular epidemiological studies to correlate fungal types found in the environmental air with types isolated from human disease.

**ITS regions for epidemiological investigations**

**Introduction**

DNA-based methods for epidemiological typing of fungal pathogens have only recently been developed [59,204,205]. The methods showing the greatest promise are RFLP analysis and RAPD analysis of the whole fungal genome. To expand this area of testing, locus-specific subtyping using a PCR assay in combination with RFLP and sequence comparisons has been accomplished with a number of rDNA targets [206]. Radford *et al.* [206] used intergenic spacer sequence variations to successfully subtype *Aspergillus fumigatus* isolated from patients and from the environment of a bone marrow transplantation unit over an extended period of time. They were able to show a correlation between environmental strains and those detected from infected patients. Discussed below are examples in which sequence variations within ITS regions have been used as a method for typing fungal strains. Most of the work using ITS–PCR for typing has focused on the evaluation of *P. carinii* by DNA sequence analysis.

**Applications**

Typing of *P. carinii* using the ITS regions as targets was originally done by Lu *et al.* [207]. They showed that enough sequence variation existed within the ITS regions to distinguish among strains of *P. carinii* isolated from BAL fluid specimens of infected patients. They subsequently developed a typing procedure using oligonucleotide probes specific for each ITS type of *P. carinii* [208]. They referred to the procedure as type-specific oligonu-
cleotide typing, where two types of ITS1 (A and B) and 3 types of ITS2 (a, b and c) variants were designated. To simplify this procedure, they subsequently developed a nested PCR method using type-specific PCR primers to distinguish ITS1-A from ITS1-B, followed by reaction with ITS2-specific probes for a, b and c strains [209]. This not only simplified the procedure, but allowed for a more distinct determination of types within P. carinii. Since this report, the same research group has expanded the sample size to include 207 P. carinii-positive specimens (from BAL fluid, BAL fluid slide smears and paraffin-embedded lung biopsy specimens) so that the number of ITS1 sequence types has now been broadened from 2 to 15, and the ITS2 sequence types have increased in number from 3 to 14 [199].

Latouche et al. [210] also compared DNA regions of P. carinii and identified the ITS regions as the most discriminatory region for analysis of the biodiversity of P. carinii. In their evaluation, they studied the thymidylate synthase gene, the 5S rDNA gene, the mt rDNA gene, and the ITS regions of BAL specimen isolates from human immunodeficiency virus (HIV)-infected patients with PCP. In a subsequent study, these same researchers typed P. carinii strains obtained from different BAL specimens and induced sputum samples during a given or recurrent episode of PCP [211]. They showed by sequence analysis of the ITS regions that de novo infection occurred in AIDS patients with recurrent PCP. This work was subsequently confirmed by Margutti et al. [212] who biotyped P. carinii strains isolated from BAL fluid samples of AIDS patients who had multiple episodes of PCP. They showed that in cases where there was a lengthy interval between PCP episodes, that the P. carinii strains were clearly different types in a majority of cases, suggesting a new infection from an exogenous source of P. carinii had occurred, as opposed to relapse from a prior strain. Tsolaki et al. [213] also investigated ITS sequence variations in P. carinii DNA detected from BAL fluid of HIV-infected patients with episodes of recurrent PCP. While evaluating the sequence variations, they too identified multiple types of P. carinii. They subsequently tested archived frozen samples of postmortem lung tissue positive for P. carinii from immunocompromised patients before the AIDS pandemic [201]. Data from this study showed that P. carinii DNA from PCP before the recognition of the AIDS pandemic was genetically similar to DNA recovered from HIV-infected individuals, indicating that mutational changes over this period of time were minimal. Furthermore, these authors performed genotyping of P. carinii using the ITS region as a target in paired samples of oropharyngeal and BAL specimens from HIV-infected patients with PCP. They showed that ITS sequence typing of P. carinii from oropharyngeal samples appeared to be a reliable alternative to BAL samples and provided a noninvasive tool for epidemiological studies [214]. Genotyping of P. carinii isolates based on nucleotide sequence variations in ITS regions of the rRNA gene have also shown that multiple genotypes may be present in one patient. Hosoya et al. [185] reported 16/24 patients had two or more P. carinii genotypes. Additionally, Helweg-Largen et al. [215] in an analysis of variations in the ITS regions in P. carinii, showed that changes in genotype can be detected in a single episode of PCP, suggesting that a patient can originally be infected with more than one P. carinii genotype. They suggested that it might be difficult to use genotyping as a genetic marker to separate new infection from the reactivation of latent infection.

Jiang et al. [75] also applied this methodology to the typing of H. capsulatum. They examined 24 isolates of H. capsulatum from a variety of locations. Variations were noted in both the ITS1 and the ITS2 regions, which allowed them to classify these isolates into 10 distinct types. They suggested that further studies were warranted to determine if sexual recombination by H. capsulatum, which occurs naturally in nature, can interfere with the ITS typing to classify this organism. Iwen et al. [216] also showed that sequence differences within the ITS regions of multiple H. capsulatum strains may be useful for epidemiological investigations, but that this intraspecies sequence diversity did not limit the ability to identify this pathogen following sequence analysis.

**Future applications**

The application of the ITS regions sequences to species identification is likely to expand based on new technologies that are being implemented. Turenne et al. [217] applied an automated fluorescent capillary electrophoresis sequencer (ABI PRISM 310 genetic analyzer, Perkin–Elmer Applied Biosystems, Foster City, California, USA) to differentiate fungi based on sequence variability of the ITS2 region alone. In this technique, the sequencer detected fluorescent-tagged amplicons, which were subsequently evaluated using the ABI PRISM 310 GeneScan analysis software to determine fragment size based on electrophoretic mobility of the sample in relation to an internal standard. The ability to combine electrophoresis with fluorescence helped overcome the problem of minimal differences in size of the amplicons produced from PCR. A number of clinically significant fungal species were tested and differentiated correctly within 7 h for a rapid diagnosis of fungemia and other invasive fungal infections using this technique.
Innovative technologies using a segment of the genome have also proven useful in clinical studies. Loeffler et al. [173] utilized a method to quantify fungal DNA using fluorescence resonance energy transfer and the Light Cycler (Roche Diagnostics, Mannheim, Germany). They employed the 18S-rDNA gene as the molecular target and were able to establish a quantitative PCR protocol for both C. albicans and A. fumigatus. Utilization of this same technology with the ITS regions as targets may prove useful to differentiate infection from colonization when evaluating those specimens that may contain normal flora fungi, such as BAL fluid.

Other applications that may be valuable in the use of ITS-PCR include denaturing high-pressure liquid chromatography (DHPLC) with reverse-phase ion-pairing chromatography for the separation of nucleotide fragments (WAVE System, Transgenomics, Omaha, Nebraska, USA) and DNA microchip array technology. DHPLC has been shown to be useful for the rapid and inexpensive fingerprinting of bacteria [218]. Application of this technology to the identification of fungal pathogens has also shown promise (P.C. Iwen, unpublished results). DNA arrays, which are widely used to study gene expression patterns and to identify new genes, have recently been applied to the area of DNA-based diagnostics [4,219]. Applying this technology to the identification of fungi has not yet been reported, but appears appropriate using the ITS regions as targets.

Conclusions

The research studies summarized in this review demonstrates that the ITS regions of the rDNA gene complex are useful as targets in molecular applications and offer a powerful tool for the identification and typing of fungi. The key aspect of this approach is the location of the ITS regions between two highly conserved genes allowing for their amplification using universal fungal primers. Direct sequence analysis, species-specific probing and RFLP analysis using the ITS target in PCR-based assays have all shown utility for the identification of fungal pathogens.

One of the most significant limitations to using the ITS region for fungal identification is the relatively few species from diverse hosts and geographical origins that have had the ITS regions evaluated. More work needs to be done to determine the degree of polymorphism both between and within fungal species. Intraspecies divergence has been reported within the ITS regions of Fusarium species, and this discordance suggests that the morphological-based taxonomic scheme for Fusarium may be incorrect and that a new classification may be required [57,58,130,220]. Conversely, ITS sequence variations among some species of fungi are minimal, making it difficult to separate species based only on sequence comparison [56,79,111,129]. In both situations, the issue is whether a reevaluation of the phenotypic positioning of these fungal species is warranted and whether ITS sequences provide the reference method. The sequence polymorphism within ITS regions nonetheless has also been shown to be sufficient for the typing of fungal species for epidemiological studies, including P. carinii and H. capsulatum [75,199,210,212,214].

In conclusion, DNA-based assays using the ITS regions as molecular targets show great potential for the diagnosis of invasive fungal infections. A simple, rapid and sensitive test that incorporates these features and that can be routinely used in the clinical mycology laboratory is an important clinical goal for the proper management of patients with an invasive fungal disease.

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