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Molecular methods for the identification of *Aspergillus* species

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Invasive aspergillosis (IA) is a leading cause of morbidity and mortality in immunocompromised hosts. In some institutions, species of *Aspergillus* less susceptible to amphotericin B than *Aspergillus fumigatus* are becoming more common, making an accurate identification of species important. However, species identification has traditionally relied on macroscopic colony characteristics and microscopic morphology, which may require several days of culture. Additional sub-culturing on specialized media may be required to induce conidia formation; in some cases conidia may never form, confounding identification. Therefore, rapid, nucleic acid-based methods that identify species of *Aspergillus* independent of morphology are now being developed to augment or replace phenotypic identification methods. The most successful methods to date have employed polymerase chain reaction (PCR) amplification of target sequences within the ribosomal RNA gene complex, including the 28S ribosomal subunit (D1-D2 region) and the internal transcribed spacers 1 and 2 (ITS1 and ITS2 regions). We therefore developed a PCR-based assay to differentiate medically important species of *Aspergillus* from one another, and from other opportunistic moulds and yeasts, by employing universal, pan-fungal primers directed to conserved ribosomal genes and species-specific DNA probes directed to the highly variable ITS2 region. Amplicons were then detected in a simple, colorimetric enzyme immunoassay format (PCR-EIA). DNA sequencing of the ITS1 and ITS2 regions and of the D1-D2 region was also conducted for the differentiation of species by comparative GenBank sequence analysis. The PCR-EIA method was found to be rapid, sensitive, and specific for the identification and differentiation of the most medically important species of *Aspergillus*. In addition, methods to identify species of *Aspergillus* by comparative GenBank sequence analysis were found to be more reliable using the ITS1 and ITS2 regions than the D1-D2 region.

Keywords *Aspergillus* species, DNA sequencing, internal transcribed spacer, molecular identification, PCR, rRNA, rDNA

Introduction

Aspergillus fumigatus remains the most frequent cause of invasive aspergillosis (IA) although at least 30 other species, primarily *A. flavus*, *A. terreus*, *A. niger*, *A. nidulans*, *A. ustus*, and *A. versicolor*, have been associated with human disease [1]. In some institutions, *A. terreus* is becoming more common and is of concern because it is less susceptible to amphotericin B than *A. fumigatus* [2–4]. *A. nidulans* has also been reported to be less susceptible to amphotericin B than *A. fumigatus* [5]. Although a rare cause of invasive disease, *A. ustus* has been reported to be resistant to

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amphotericin B while remaining susceptible to itraconazole [6]. *A. ustus* has also been reported to be less susceptible to voriconazole than *A. fumigatus* [7]. Therefore, accurate and timely identification of species is important for the management of IA as well as for surveillance and other epidemiological purposes.

Identification of species of *Aspergillus* has traditionally relied on macroscopic colony characteristics and microscopic morphology. Unfortunately, several days of culture may be required for the development of specific phenotypic characteristics and conidia formation that guide identification. Failure to form conidia on ordinary culture media may require colonies to be further sub-cultured on specialized media to induce spore formation. In some cases, conidia never develop, impeding identification. Therefore, rapid, nucleic acid-based methods that identify the species of *Aspergillus*, independent of morphology, are now being developed to augment or replace phenotypic identification.

We describe here molecular methods developed in our laboratory for the differentiation of the most medically important species of *Aspergillus* from one another, as well as from other moulds and yeasts, by a polymerase chain reaction-enzyme immunoassay (PCR-EIA) and by sequencing and comparative GenBank sequence analysis of ribosomal DNA regions.

Materials and methods

Microorganisms

Isolates of each *Aspergillus* species, including type culture strains, were grown on Czapek-Dox agar (Difco Laboratories, Detroit, Mich.) for up to 14 days at 25°C, and for up to one week at 37°C, to confirm their purity and species identity using traditional macroscopic and microscopic criteria and to generate sufficient growth for DNA extraction.

DNA extraction and amplification

Fungal growth was transferred from the surface of a single agar plate into a pre-cooled (−20°C) sterile ceramic mortar, overlaid with liquid nitrogen, and ground with a sterile ceramic pestle into a fine powder. Two ml of buffer G-2 (Genomic DNA buffer set; Qiagen, Valencia, Calif.) containing RNase (200 μg/ml; Sigma Chemical Company, St. Louis, Mo.) was added to suspend the powder and the suspension was transferred into a clean test tube. Forty-five microliters of proteinase K solution (20 mg/ml stock solution; Sigma) was added and the suspension was incubated with intermittent agitation for 3 h at 55°C. The suspension was centrifuged at 21,500 × *g* for 10 min, the supernatant was transferred into a clean test tube, and DNA was extracted and purified using Genomic-tip 20/G columns (Qiagen) according to the manufacturer's instructions. Two and one-half microliters of glycogen solution (20 mg/ml; Gentra Systems, Minneapolis, Minn.) was added to the eluted DNA which was then precipitated by standard methods using isopropanol and ethanol. DNA was resuspended in 60 μl of DNA rehydration buffer (PureGene kit, Gentra Systems) and stored at −20°C until used.

Detection of PCR amplicons by enzyme immunoassay (PCR-EIA)

Universal fungal primers ITS3 (5′ GCA TCG ATG AAG AAC GCA GC) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC), directed to the internal transcribed spacer 2 (ITS2) region of ribosomal DNA (rDNA) (Fig. 1), were employed to amplify DNA from all species of *Aspergillus* tested. PCR amplicons were then detected colorimetrically as previously described [8].

DNA sequencing

Semi-nested PCR, using universal fungal primers ITS1 (5′ TCC GTA GGT GAA CCT GCG G) and D2R (5′ TTG GTC CGT GTT TCA AGA CG), followed by primers D1 (5′ GCA TAT CAA TAA GCG GAG GA) and D2R, generated the 28S rDNA D1-D2 region amplicons for sequencing. Universal fungal primers

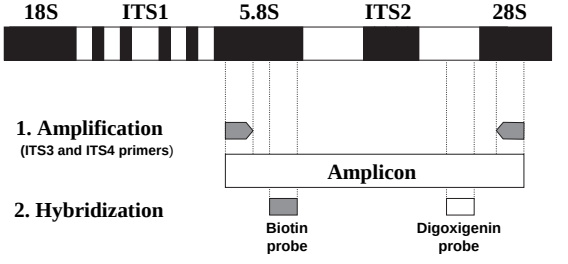

Fig. 1 Detection of ITS2 rDNA amplicons by PCR-EIA [8]. Binding sites for universal fungal primers ITS3 and ITS4 in the conserved 5.8S and 28S regions of rDNA are shown in step 1. Hybridization of biotinylated and digoxigenin-labeled probes, directed to the amplicons produced after PCR amplification of the ITS2 rDNA region, is shown in step 2. After hybridization to the amplicon, the biotin probe is captured onto streptavidin-coated microtiter plate wells and the digoxigenin probe, hybridized adjacent to the biotin probe, is detected spectrophotometrically (A_{650nm}) after addition of horseradish peroxidase-conjugated anti-digoxigenin antibodies, H_2O_2, and a colorimetric substrate.

ITS5 (5′ GGA AGT AAA AGT CGT AAC AAG G) and ITS2 (5′ GCT GCG TTC TTC ATC GAT GC) or ITS3 and ITS4 were used to PCR amplify rDNA regions ITS1 and ITS2, respectively. PCR amplification was conducted in a GeneAmp model 9700 thermal cycler (Perkin Elmer Applied Biosystems, Foster City, Calif.) as described previously [9]. All PCR products were purified before DNA sequence analysis using a QIAquick PCR Purification kit (Qiagen). Purified amplicons were then sequenced on both strands using the same primers as described above and BigDye Terminator Cycle Sequencing Ready Reaction kits (Perkin Elmer Applied Biosystems) as recommended by the manufacturer [9]. Products were analyzed on an automated capillary DNA sequencer (ABI Prism 310 Genetic Analyzer, Perkin Elmer Applied Biosystems) according to the manufacturer′s directions.

Results

Universal fungal primers and species-specific DNA probes for the identification of aspergilli

Species-specific probes for seven species of *Aspergillus* (*A. flavus*, *A. fumigatus*, *A. nidulans*, *A. niger, A. terreus*, *A. ustus*, and *A. versicolor*) were designed after DNA sequencing and alignment of ITS2 rDNA regions from multiple, authenticated culture collection strains [8,9]. Unique signature sequences within each species were identified and DNA probes were designed from these regions (Fig. 2). Each of the seven species probes were specific for their homologous target DNA (Table 1) and did not cross-react with DNA from any of 34 species of *Acremonium*, *Candida*, *Exophiala*, *Fusarium*, *Mucor, Paecilomyces*, *Penicillium*, *Rhizopus*, *Rhizomucor*, *Scedosporium*, or *Sporothrix* species that were tested [8]. These probes did not cross-react with DNA from any of eight other species of *Aspergillus* tested, with two exceptions. The *A. nidulans* probe cross-reacted with *A. ustus* DNA, and the *A versicolor* probe gave minor cross-reactivity with *A. candidus* DNA (i.e., the *A. versicolor* probe gave a ten-fold lower colorimetric reading with *A. candidus* DNA than with *A. versicolor* DNA). In the first instance, although the *A. nidulans* probe cross-reacted with *A. ustus* DNA, the *A. ustus* probe did not cross-react with *A. nidulans* DNA (Table 1). Therefore, these two species could be differentiated by a process of elimination. DNA sequence analysis demonstrated the existence of unique *A. candidus* signature sequences upstream from the *A. versicolor* probe region that could easily be used to design a probe specific for *A. candidus* DNA [9].

The limit of sensitivity for the PCR-EIA system was found to be 0.5 pg of target DNA (equivalent to approximately 1 to 10 conidia) compared to 5 pg of DNA detected after agarose gel electrophoresis and ethidium bromide staining of PCR products [8]. The limit of test sensitivity was similar to that reported by others using PCR amplification systems and Southern blot detection of amplicons [10]. Therefore, the combined use of universal, fungus-specific PCR primers that target conserved, multi-copy genes present in all fungal genera, followed by specific DNA probes to identify a given species, was shown to be highly sensitive and specific. The major advantages of the PCR-EIA identification system, compared to conventional phenotypic identification methods, were:

1. it was rapid (this test could be completed in a single day);
2. it did not require species of *Aspergillus* to form specialized identifying structures such as conidia;
3. small amounts of DNA target could be detected (picogram quantities);
4. DNA probes could be easily and reproducibly synthesized and stored ready for use;
5. DNA probes were very specific;
6. interpretation of the results were objective (colorimetric, spectrophotometric readout);
7. the detection system has the potential to be easily automated.

DNA sequencing and comparative GenBank data analysis

Although the PCR-EIA method was found to be highly sensitive and specific, probes were designed to detect only seven of the 30 known species of *Aspergillus* associated with human disease. It was hypothesized that DNA sequence analysis, using universal fungal primers and comparative GenBank searches, might provide specific identification of a larger number of *Aspergillus* species than DNA probes. However, this would require use of appropriate DNA target regions that display sufficient interspecies sequence variation, without excessive intraspecies variation. DNA target regions that have been examined for such purposes include an aflatoxin pathway regulatory gene [11,12], the β-tubulin gene [13], and various ribosomal RNA (rRNA) regions [14–17]. In addition, the variable regions at the 5′ end of the 28S rRNA gene (the D1-D2 region) and the internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) of rDNA have each been examined by others as sequencing targets [18–22].

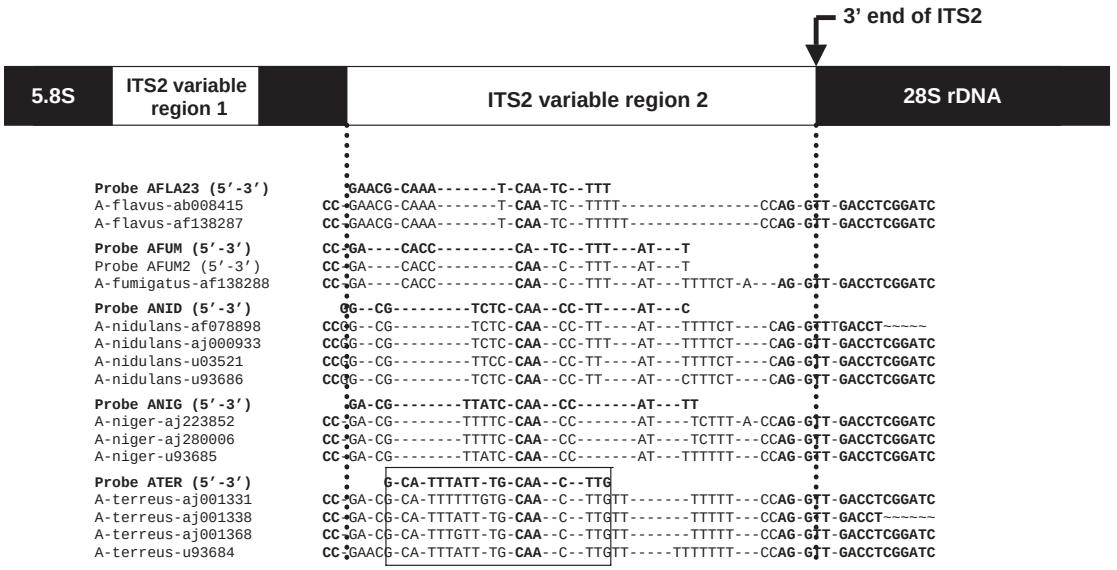

Fig. 2 Design of *Aspergillus* species-specific oligonucleotide probes for PCR-EIA. DNA sequencing of the ITS2 rDNA region for selected *Aspergillus* species was conducted and sequences were aligned and compared [9]. Unique signature sequences (i.e., those that were recognized to occur consistently among strains of a given species) located at the 3' end of the ITS2 variable region 2 were used to design species-specific oligonucleotide probes. For example, the box at the bottom of the figure identifies a signature sequence, unique to strains of *A. terreus,* that was used to design an *A. terreus*-specific PCR-EIA probe.

However, because there had been no systematic assessment or comparison of the rDNA regions for their utility as diagnostic targets for the identification of medically important species of *Aspergillus*, we sequenced the ITS1, ITS2, and D1-D2 rDNA regions of 13 medically important species (*A. candidus*, *A. [Eurotium] chevalieri*, *A. [Fennellia] flavipes*, *A. flavus*, *A. fumigatus*, *A. granulosus*, *A. [Emericella] nidulans*, *A. niger*, *A. restrictus*, *A. sydowii*, *A. terreus*, *A. ustus*, and *A. versicolor*). Sequencing results were used to examine amplicon length, to conduct pairwise nucleotide sequence comparisons and DNA alignments, and to perform comparative GenBank data analyses for the identification and differentiation of the species under study [9].

Analysis of DNA sequence length polymorphisms

No significant differences in the length of the D1-D2 region were observed among any of the 13 species of *Aspergillus* examined (all species were 542 bp in length except for *A. nidulans* which was 543 bp in length) [9]. In contrast, sequences for the ITS1 region ranged in length from 142 nt (*A. chevalieri*) to 186 nt (*A. terreus*). Sequence length was more variable among the aspergilli in the ITS1 region than in the D1-D2 region; i.e., in the ITS1 region, five of the 13 species studied demonstrated a unique sequence length (*A. candidus*, *A. chevalieri*, *A. granulosus*, *A. nidulans*, and *A. terreus*) compared to only one species (*A. nidulans*) in the D1-D2 region. Identical or similar sequence lengths were observed for *A. candidus*, *A. flavus*, and *A. restrictus*

Table 1 Specificity of ITS2 region oligonucleotide probes to detect *Aspergillus* species DNA by PCR-EIA. Adapted from de Aguirre *et al*. [8]

Target DNA	Mean A_{650nm} using oligonucleotide probe for:						
	A. flavus	*A. fumigatus*	*A. nidulans*	*A. niger*	*A. terreus*	*A. ustus*	*A. versicolor*
A. flavus	1.73	0[a]	0	0	0	0	0
A. fumigatus	0	2.37	0	0	0	0	0
A. nidulans	0	0	0.87	0	0	0	0
A. niger	0	0	0	1.24	0	0	0
A. terreus	0	0	0	0	1.51	0	0
A. ustus	0	0	0.55	0	0	1.41	0
A. versicolor	0	0	0	0	0	0	1.64

[a] Mean $A_{650nm} \pm$SE for all heterologous *Aspergillus* species DNA tested: 0.004 ± 0.003.

(180–181 nt), *A. flavipes*, *A. fumigatus*, *A. niger*, and *A. terreus* (184–186 nt), and *A. granulosus*, *A. nidulans*, *A. sydowii*, *A. ustus*, and *A. versicolor* (153–156 nt) [9].

In comparison, ITS2 sequences varied in length from 160 nt (*A. flavipes*) to 177 nt (*A. terreus*). Sequence length was slightly less variable among the *Aspergillus* spp. in the ITS2 than in the ITS1 region but was more so than in the D1-D2 region. For example, three of the 13 species examined demonstrated a unique sequence length in the ITS2 region (*A. chevalieri, A. granulosus,* and *A. terreus*), and five shared identical sequence length with at least one strain from each of the 13 species examined (e.g., *A. chevalieri* and *A. versicolor,* 167 nt). Identical or similar sequence lengths were observed for *A. candidus*, *A. chevalieri*, *A. flavus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *A. sydowii*, and *A. versicolor* (167–169 nt), and among *A. granulosus*, *A. restrictus*, and *A. ustus* (170–172 nt). However, combining data for both the ITS1 and ITS2 regions, including or excluding the intervening conserved 5.8S region, allowed all 13 species examined to be differentiated from one another (ITS1+ITS2 sequence length). The only exceptions were for *A. candidus* (505 nt) and one non-type strain of *A. flavipes* (*A. flavipes* I; 505 nt) and for a non-type strain of *A. nidulans* (*A. nidulans* II, 480 nt) and *A. sydowii.* These data reflect comparisons among the 13 species of *Aspergillus* selected. Sequence length comparisons among molecular siblings of these species may prove less discriminatory and this will become more obvious below. In general, the sequence length of any ribosomal region alone could not be reliably used to differentiate among all species examined. However, the greatest degree of discrimination among species was obtained using combined ITS1 and ITS2 sequence data [9].

Pairwise nucleotide sequence comparisons

Pairwise nucleotide sequence comparisons within the D1-D2 region demonstrated differentiation of the most medically important species to at least the group level, although some closely related species shared greater than 99% sequence identity (i.e., *A. granulosus* versus *A. ustus* and *A. sydowii* versus *A. versicolor*). In contrast, none of the species examined demonstrated sequence identities of greater than 99% in either the ITS1 or ITS2 regions, irrespective of phylogenetic relatedness [9]. Interspecies sequence identities ranged from 91.9% to 99.6% for the D1-D2 region, from 57.4% to 98.1% for the ITS1 region, and from 75.6% to 98.3% for the ITS2 region (Table 2).

Intraspecies variability was minimal in the D1-D2 region; strains differed by 0 nt for all species examined except for strains of *A. flavipes, A. sydowii*, and *A. nidulans,* which differed by 1–2 nt from their respective type strains. In contrast, sequence variability from the type strain of 1–14 nucleotides (1 nt, *A. nidulans*; 14 nt, *A. ustus*) occurred among strains of *A. niger*, *A. flavipes*, *A. ustus*, *A. sydowii*, and *A. nidulans* in the ITS1 region. Differences of 1–8 nucleotides (1 nt, *A. niger* and *A. nidulans*; 8 nt, *A. flavipes*) occurred among strains of these same species in the ITS2 region (Table 2). Therefore, the rank order for the greatest to least interspecies differences (most species variability) was ITS1 > ITS2 > D1-D2 and the rank order for the least to greatest intraspecies divergence (least strain variability) was D1-D2 < ITS2 < ITS1. It is not known if the observed intraspecies differences are an artifact of current taxonomy or if these strains represent true sequevars (i.e., a sequevar is a group of strains with sequences that are identical to one another but which vary from those of other strains within the same species).

Table 2 Comparative variation between species and within species as assessed by pairwise nucleotide sequence alignment of rDNA regions

rDNA region	Range in:	
	Interspecies sequence similarities (%)[a]	Intraspecies sequence differences (nt)[b]
D1-D2	91.9%–99.6%	0–2
ITS2	75.6%–98.3%	0–8
ITS1	57.4%–98.1%	0–14

[a] Percent sequence identity among different species of *Aspergillus*.
[b] Variability between strains within a given species as measured by the number of nucleotide (nt) differences from the type strain sequence.

DNA sequence alignments and comparative GenBank data searches

DNA sequence alignments revealed greater sequence variability among species of *Aspergillus* in the D2 than in the D1 region of rDNA whereas even greater differences were observed in five variable ITS1 and two variable ITS2 regions [9]. Comparative GenBank data searches demonstrated that D1-D2 sequences rarely allowed unambiguous differentiation of closely related species; 10 of 13 *Aspergillus* species examined showed $\leq$1 nt sequence divergence from organisms catalogued in GenBank as a different species. In contrast, only five of the species examined exhibited $\leq$1 nt divergence with a different species in their ITS1 or ITS2 sequences. For example, a GenBank query using *A. flavus* sequences matching those of the type strain for *A. flavus* in all three rDNA regions examined gave 100% identity with five molecular siblings in the

Table 3 Molecular siblings identified for *Aspergillus flavus* by a comparative GenBank database search. Adapted from Hinrikson *et al*. [9][a]

Query sequence[a]	Molecular siblings[b]	Maximum sequence identity as percent of target[c]		
		D1-D2	ITS1	ITS2
A. flavus	*A. oryzae*	100.0	100.0	100.0
	A. parasiticus	100.0	97.8	97.7
	A. sojae	100.0	97.8	97.7
	A. tamari	99.8	95.6	97.7
	A. subolivaceus	100.0	NA[d]	NA
	A. terricola	100.0	NA	NA
	A. flavofurcatus	99.8	NA	NA

[a] Reference strain with identical D1-D2, ITS1, and ITS2 sequences compared to the type strain.
[b] Organisms assigned in GenBank to a different species than query sequence although exhibiting identical or very similar (>99% identity) sequences in at least one ribosomal region examined.
[c] Comparative identity between query sequence and reference sequences of molecular siblings in the GenBank database.
[d] NA = no corresponding reference data available for this molecular sibling in the ITS region at the time of this study.

D1-D2 region and only one molecular sibling in each of the ITS1 and 2 regions (Table 3; molecular siblings are defined in footnote b). Furthermore, only four of the species examined (*A. chevalieri*, *A. flavus*, *A. nidulans*, and *A. niger*) shared identical sequences with other species in both ITS regions [9]. The current GenBank database does not contain ITS sequences for all species (designated in Table 3 as "NA" – not currently available), and major improvement of GenBank data in terms of sequence quality and annotation is needed. Therefore, at present, the identification of medically important aspergilli using comparative GenBank sequence analysis seems more reliable using combined ITS1-ITS2 sequence data rather than D1-D2 sequences. It is important to note that the sequence identities reported here are those obtained with a limited number of strains. It is possible that sequence identities between medically important species and their molecular siblings may be as high as 100% in any of the rDNA regions examined for strains that were not examined in this study. Also, because molecular taxonomic structure is under construction, it is not known if changes in traditional phenotypic classification may occur in the future, which would alter the interpretation of the above results.

Discussion

Species of *Aspergillus* shown to be less susceptible to amphotericin B than *A. fumigatus* are becoming important in some institutions [2,3,6]. For example, *A. terreus* has been reported to account for 16% of IA cases in hematopoietic stem cell transplant recipients and was responsible for almost 12% of cases in solid organ transplant patients [23]. Therefore, rapid and accurate identification of the infecting species has become particularly important for the implementation of appropriate antifungal therapy, as well as for epidemiologic purposes. Currently, phenotypic methods for the identification of the most common aspergilli remain useful in the clinical microbiology laboratory. Less common species are referred to reference laboratories for identification as are atypical or non-conidiating isolates. Because species of *Aspergillus* are ubiquitous, not every isolate received in a clinical laboratory needs to be identified to species. However, if an isolate is linked to a high-risk patient or is collected in conjunction with suspected invasive disease, species identification may help guide clinical management. In addition, information about species identity is important for epidemiologic purposes, such as for outbreak investigations, surveillance for the emergence of new species causing disease, and an accurate determination of incidence rates. In such cases, species-specific probes, particularly in a microarray format, would be useful for the rapid screening of a large number of samples.

Microarray formats provide the advantages of requiring only a small amount of PCR amplicon for a large amount of data retrieved (hundreds of probes can be included in a single run), data retrieval and analysis is rapid and can be automated, and the cost can be minimal if glass slides are manufactured in-house. A prototype microarray, constructed in our laboratory for the differentiation of multiple species of *Aspergillus,* as well as for the differentiation of species of *Candida, Fusarium*, *Scedosporium* and some zygomycetes, is currently being evaluated and developed for commercialization. Microarrays have been designed elsewhere for identifying bacteria [24] and are under development for identifying and differentiating species of *Candida* [25]. Both the D2 region of 28S rDNA [26] and the ITS1 and ITS2 regions [8,27] have been used to design species-specific diagnostic probes. Probes directed to

the D2 region identify most species to the traditional (phenotypic) or phylogenetic group level as defined by Raper and Fennell [28] and Peterson [18], respectively, whereas the ITS probes may identify distinct species. Some intraspecies variability was noted in the ITS regions, but it is not known if this represents differences between phenotypic and molecular taxonomic structure as reported for other genera [29] or if true sequevars exist within a species. In any case, both probe methods and microarray methods require known sequence information for the design of specific probes for a given detection format or array.

A broader approach, which would capture known as well as unknown species, would be to apply universal DNA sequence analysis. However, universal DNA sequence analysis has several limitations, as demonstrated by our work, particularly when public databases are being used as the source of reference information. Currently available public databases are not refereed and their quality is solely dependent on the accuracy of the submitter. Commercial databases are becoming available but are currently expensive and incomplete or are not in concordance with phenotypic identification results. A recent study reported only 50%–60% of the molecular identifications were concordant with the results of phenotypic methods [30]. The lack of concordance between methods in this study was partly attributed to the incompleteness of the sequence database. Other questions, concerning the impact of molecular identification on traditional taxonomic structure, remain unresolved at present. Complete evaluation of future databases will need to rely on a uniform, standardized molecular taxonomic structure. A true species definition will be needed and may not be easily assessed on the basis of percent sequence homology for ribosomal genes alone. Other methods, or combinations of methods [31,32], such as genomic DNA-DNA complementarity measurements, isoenzyme studies, and DNA sequence analysis of multiple genes, may be required. Taxonomic structure will very much depend on what DNA region or regions are chosen for sequence analysis. For example, the more conserved 18S and 28S regions tend to combine more closely related species into the same taxonomic group, whereas more variable regions such as the ITS regions tend to split groups into distinct species. Ideally, multiple regions, reflecting the true evolution of a species, will be discovered and all regions will provide concordant findings. Different molecular targets may provide varying degrees of differentiation, depending on the organisms under study, and targets may need to be tailored for optimum results. Sequences for the rRNA intergenic spacer (IGS) and ITS regions [32] or sequences for the 28S rRNA gene, a portion of the elongation factor-1 alpha gene, and a combination of both of the latter genes have been used to differentiate species of *Fusarium* [33–35]. Other molecular targets, or even other genotyping methods, may be necessary for optimal identification and differentiation of some species. In addition, the difficulty of defining a species is made all the more complex because many of the organisms under study have no known sexual state. Species definitions based on a biologic concept (genetic crosses) become impossible or, at the very least, impractical.

Despite the limitations described above, molecular methods for the identification of species of *Aspergillus* and of other medically important fungi will no doubt expand rapidly in the near future. Automated technologies combining extraction of microbial DNA from clinical materials, DNA amplification, and amplicon detection within a single, closed system are currently under commercial development for fungal pathogens [36] and automated systems are in use in many clinical laboratories for the detection of viral and bacterial pathogens [37,38]. Pan-microbial microarrays, particularly syndromic arrays (i.e., meningitis, respiratory, or septicemia arrays) for fungal as well as bacterial agents would provide the greatest amount of information and require the least amount of target DNA per assay. Such arrays are already in development for the identification of the most common bacteria causing urinary tract [39] and bloodstream infections [40]. At a minimum, the PCR-EIA system has been shown to have clinical utility for the detection of DNA from species of *Candida* recovered from blood culture bottles [41] and for the detection of DNA from species of *Aspergillus* recovered from tissue in an experimental model of aspergillosis [8]. At the same time, larger and more accurate DNA databases emanating from public, private, and commercial sectors will become available. Improved DNA sequence information, in conjunction with other identification methods, will enable the establishment of a standardized, objective taxonomic structure for the classification of fungi. The impact of such advances in molecular identification methods on clinical outcome is yet to be determined but should ultimately lead to improved diagnosis, therapy, and management of fungal diseases, including invasive aspergillosis.

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