

## Identification of *Aspergillus* Species Using Internal Transcribed Spacer Regions 1 and 2

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***Aspergillus* species are the most frequent cause of invasive mold infections in immunocompromised patients. Although over 180 species are found within the genus, 3 species, *Aspergillus flavus*, *A. fumigatus*, and *A. terreus*, account for most cases of invasive aspergillosis (IA), with *A. nidulans*, *A. niger*, and *A. ustus* being rare causes of IA. The ability to distinguish between the various clinically relevant *Aspergillus* species may have diagnostic value, as certain species are associated with higher mortality and increased virulence and vary in their resistance to antifungal therapy. A method to identify *Aspergillus* at the species level and differentiate it from other true pathogenic and opportunistic molds was developed using the 18S and 28S rRNA genes for primer binding sites. The contiguous internal transcribed spacer (ITS) region, ITS 1–5.8S–ITS 2, from referenced strains and clinical isolates of aspergilli and other fungi were amplified, sequenced, and compared with non-reference strain sequences in GenBank. ITS amplicons from *Aspergillus* species ranged in size from 565 to 613 bp. Comparison of reference strains and GenBank sequences demonstrated that both ITS 1 and ITS 2 regions were needed for accurate identification of *Aspergillus* at the species level. Intraspecies variation among clinical isolates and reference strains was minimal. Sixteen other pathogenic molds demonstrated less than 89% similarity with *Aspergillus* ITS 1 and 2 sequences. A blind study of 11 clinical isolates was performed, and each was correctly identified. Clinical application of this approach may allow for earlier diagnosis and selection of effective antifungal agents for patients with IA.**

*Aspergillus* species are associated with allergic bronchopulmonary disease, mycotic keratitis, otomycosis, nasal sinusitis, and invasive infection. The most severe disease caused by aspergilli occurs in immunocompromised patients, with invasive pulmonary infection followed by rapid dissemination. The frequency of invasive mold infections has increased in recent years due to the increasing number of patients receiving aggressive chemotherapy regimens and immunosuppressive agents (2). The nonspecific symptoms and the lack of rapid diagnostic assays to detect these infections have been major problems in treating patients with invasive disease, particularly those with invasive aspergillosis (IA). Early recognition of invasive fungal infection and treatment with appropriate antifungal therapy are key to reducing the mortality associated with disseminated disease (25). The mortality rate for bone marrow transplant patients with pulmonary IA is greater than 70% (5, 15). Due to the typically long time required for identification of a mold using standard culture procedures, most patients with suspected disease are treated empirically with amphotericin B (AmB). Resistance to AmB as well as itraconazole has been reported for some *Aspergillus* species although the number of isolates studied in each case was limited (14, 16).

Unfortunately, the identification of aspergilli based on morphological methods requires adequate growth for evaluation of colony characteristics and microscopic features. A culture time of 5 days or more is generally required for identification of anamorphic forms of *Aspergillus*. There are more than 180 species in the *Aspergillus* genus, although 3, *Aspergillus flavus*, *A. fumigatus*, and *A. terreus* account for the vast majority of IA

infections. *A. nidulans*, *A. niger*, and *A. ustus* are rarely encountered as causes of invasive disease (18).

Various molecular approaches have been used for the detection of *Aspergillus* from environmental and clinical samples (3, 6, 27). Targets for the genus level detection of *Aspergillus* have included the 18S rRNA gene, mitochondrial DNA, the intergenic spacer region, and the internal transcribed spacer (ITS) regions. The ITS regions are located between the 18S and 28S rRNA genes and offer distinct advantages over other molecular targets including increased sensitivity due to the existence of approximately 100 copies per genome. The rRNA gene for 5.8S RNA separates the two ITS regions. The sequence variation of ITS regions has led to their use in phylogenetic studies of many different organisms (9, 26). Most recently, Turenne et al. have proposed the use of ITS amplicons of different lengths for identification of *Aspergillus* species by capillary electrophoresis (CE) (23).

The goal of this study was to compare the ITS 1 and 2 nucleotide sequences of clinically important *Aspergillus* species and determine whether sufficient variability existed for identification to the species level. The majority of GenBank ITS sequences available prior to this study were either incomplete or were generated from nonreferenced isolates. Therefore, the ITS sequences of referenced pathogenic *Aspergillus* species and other opportunistic fungi were determined. A standardized method was developed for identification, and the ability of this approach to identify pathogenic *Aspergillus* strains was evaluated in a blind clinical study.

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### MATERIALS AND METHODS

**Cultures for analysis.** Referenced cultures of *Aspergillus* species obtained from the American Type Culture Collection (ATCC) included *A. flavus* ATCC 16883, *A. fumigatus* ATCC 36607, *A. nidulans* ATCC 10074, *A. niger* ATCC 16888, and

*A. terreus* ATCC 16792. *A. ustus* UAMH 9479 was obtained from the University of Alberta Microfungus Collection and Herbarium. Isolates of *Aspergillus* species from cases of IA were obtained from patient samples catalogued at the University of Nebraska Medical Center (UNMC) and inventoried in the Invasive Molds Infection (IMI) database. Morphological identification of clinical isolates to the species level was accomplished using established procedures including microscopic and macroscopic characteristics. Additional fungal species selected for sequence comparison with *Aspergillus* reference strains are listed in Table 4.

**Culture preparation and DNA extraction.** Extraction of DNA from fungi was performed following the needle inoculation of 50 ml of Sabouraud dextrose (SAB) broth (Difco Laboratories, Detroit, Mich.) with conidia from a 7-day culture in SAB agar and incubation for 72 h at 30°C. The hyphae were recovered on a 0.45- $\mu$ m-pore-size filter and washed with sterile saline. Aliquots of the fungal hyphae were stored frozen at -70°C until use. Prior to lysis, the hyphae were thawed and suspended in 400  $\mu$ l of DNA extraction buffer (1 mM EDTA [pH 8.0], 1% sodium dodecyl sulfate, 10 mM Tris-HCl [pH 7.6], 100 mM NaCl, 2% Triton X-100) as described by Van Burik et al. (24). Microcentrifuge tubes (1.5 ml) containing hyphae and buffer were sonicated in a water bath (Branson; model 2210) for 15 min, followed by heating at 100°C for 5 min. Following lysis, DNA was purified using the QIAamp blood kit (Qiagen Inc., Valencia, Calif.) and protocols for crude cell lysates supplied by the manufacturer. Following extraction, the purified DNA was stored at 4°C until tested.

**Primers.** Two oligonucleotide fungal primers described by White et al. were used for amplification (26). The ITS region primers (ITS 1, 5'-TCC GTA GGT GAA CCT GCG G-3'; ITS 4, 5'-TCC TCC GCT TAT TGA TAT G-3') make use of conserved regions of the 18S (ITS 1) and the 28S (ITS 4) rRNA genes to amplify the intervening 5.8S gene and the ITS 1 and ITS 2 noncoding regions. Primers were synthesized by the UNMC, Eppley Molecular Biology Core Laboratory.

**PCR amplification.** The PCR assay was performed with 5  $\mu$ l of test sample in a total reaction volume of 50  $\mu$ l consisting of PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl; 0.1 mM (each) dATP, dGTP, dCTP, and dTTP; 1.5 mM MgCl<sub>2</sub>; 0.3  $\mu$ M (each) primer; and 1.5 U of PlatinumTaq high-fidelity DNA polymerase (Gibco BRL, Life Technologies, Gaithersburg, Md.). Forty cycles of amplification were performed in a Stratagene Robocycler model 96 thermocycler after initial denaturation of DNA at 95°C for 4.5 min. Each cycle consisted of a denaturation step at 95°C for 30 s, an annealing step at 50°C for 30 s, and an extension step at 72°C for 1 min, with a final extension at 72°C for 3 min following the last cycle. After amplification, the products were stored at 4°C until used.

**Cloning of PCR products.** Amplicons were separated by agarose gel electrophoresis, purified, and ligated into the pCR 2.1 plasmid vector using the Original TA cloning kit (Invitrogen, San Diego, Calif.). Competent INVaF' One Shot cells were transformed using standard protocols. Colonies were isolated and purified with a Qiagen miniprep spin kit according to the manufacturer's protocols. An aliquot of purified plasmid was digested with *Eco*RI endonuclease (New England Biolabs, Beverly, Mass.) and screened by agarose gel electrophoresis for a 300-bp doublet, indicating the presence of the *Eco*RI cleavage site GAATTC within the 5.8S sequence. Selected plasmids were submitted to the Eppley Molecular Biology Core Laboratory for automated dye termination sequencing.

**DNA sequencing.** DNA sequencing was performed at the Eppley Molecular Biology Core Laboratory on a Perkin-Elmer/ABI model 373 DNA sequencer with protocols supplied by the manufacturer. For the sequencing of cloned fragments, both strands of the plasmid containing the fungal insert were sequenced with universal M13 forward and reverse sequencing primers. For direct sequencing of noncloned amplicons, PCR products were directly sequenced using the ITS 1 and ITS 4 PCR primers. The resultant nucleotide sequences were aligned with the MacVector sequence analysis software, version 6.5 (Oxford Molecular Group, Inc., Campbell, Calif.), alignment application.

**Sequence analysis.** Sequence comparisons of referenced strains and clinical isolates listed in Fig. 1 and Table 3 were made using MacVector, version 6.5, software (Oxford Molecular Group, Inc.) and the Clustal W alignment algorithm. Intraspecies sequence similarity and variation for isolates listed in Table 2 were determined by the MacVector software and were visually confirmed using pairwise nucleotide alignments. Sequences from referenced isolates were aligned to complete or partial ITS sequences available in GenBank after submission of sequence data from this study. Comparison of sequences from referenced isolates, clinical isolates, and GenBank sequences was performed using a nongapped, advanced BLAST search (1). The similarities of the sequences were determined with the expectation frequency minimized to 0.0001. Sequences were not filtered for low complexity.

**Clinical isolate identification study.** Eleven isolates of various *Aspergillus* species previously identified by the UNMC Mycology Laboratory were selected by one of us (P.I.) and inoculated onto SAB agar and incubated at 30°C for 24 h. There were three *A. fumigatus* isolates, two *A. flavus* isolates, one *A. ustus* isolate, two *A. terreus* isolates, two *A. niger* isolates, and one *A. nidulans* isolate. The plates were coded and presented for processing by a second person (T.H.). An approximately 2-mm<sup>2</sup> section of the agar at the site of inoculation was taken for DNA extraction and amplification. The amplicons were purified using the Qiagen PCR purification kit and sequenced directly. Sequence analysis of *Aspergillus* specimens was performed using an advanced, nongapped BLAST search with expectation frequency set to 0.0001 and no filtering for low complexity. The search was performed following the deposition and acceptance of sequences

TABLE 1. *Aspergillus* species PCR products

<i>Aspergillus</i> sp.	Source	Size (bp) <sup>a</sup>
<i>A. flavus</i>	ATCC 16883	595
<i>A. flavus</i>	Clinical isolate	595
<i>A. fumigatus</i>	ATCC 36607	596
<i>A. fumigatus</i>	Clinical isolate	598
<i>A. nidulans</i>	ATCC 10074	565
<i>A. nidulans</i>	Clinical isolate	569
<i>A. niger</i>	ATCC 16888	599
<i>A. niger</i>	Clinical isolate	599
<i>A. terreus</i>	ATCC 16792	609
<i>A. terreus</i>	Clinical isolate	613
<i>A. ustus</i>	UAMH 9479	570
<i>A. ustus</i>	Clinical isolate <sup>b</sup>	570

<sup>a</sup> Includes the complete ITS 1–5.8S–ITS 2 regions and portions of the 18S (30 bp) and 28S (59 bp) rRNA genes.

<sup>b</sup> Deposited into the ATCC as ATCC 201953.

from referenced isolates into GenBank. Species identification was determined from the highest bit score of the species listed from the BLAST search. The amount of time from submission of the culture plates to identification was determined.

**Nucleotide sequence accession numbers.** The ITS 1–5.8S–ITS 2 gene complex sequences of referenced *Aspergillus* species not previously available within the National Center for Biotechnology Information GenBank or EMBL databases were submitted to GenBank. The assigned sequence accession numbers are as follows: *A. flavus* (ATCC 16883), AF138287; *A. fumigatus* (ATCC 36607), AF138288; *A. niger* (ATCC 16888), AF138904; *A. terreus* (ATCC 16792), AF138290; *A. ustus* (ATCC 201953), AF157507; *A. nidulans* (ATCC 10074) (accepted into GenBank as *Emericella nidulans*), AF138289. Sequences from other fungal species also deposited into GenBank are listed in Table 4.

## RESULTS

**Analysis of the ITS regions.** Amplification of the ITS 1–5.8S–ITS 2 regions from the six clinically relevant *Aspergillus* strains generated PCR products ranging in size from 565 to 613 bp (Table 1). Sequencing was first performed on cloned amplicons and then repeated using direct sequencing of PCR products, with comparisons between results from both methods made. Although a *Taq* polymerase with proofreading capability was used in the generation of amplicons, an examination for potential variation in sequence due to random base changes introduced by the amplification process was made. Two clones from each reference strain for each species were sequenced. The sequence of cloned PCR products varied by no more than two nucleotides from the sequence of amplicons directly sequenced. Minimal differences in amplicon length between referenced and clinical strains of the same species were seen.

Alignment of contiguous fungal sequences demonstrated that both single-nucleotide differences and short lengths of sequence diversity due to insertions or deletions existed in the ITS 1–5.8S–ITS 2 regions among the pathogenic *Aspergillus* species (Fig. 1). The ITS 1 region displayed more interspecies variation than the ITS 2 region, with approximately four separate variable regions. ITS 2 contained two variable regions ranging from 6 to 10 bp in length. A matrix analysis of the sequence similarity between ITS 1 and 2 sequences of the referenced *Aspergillus* species is depicted in Table 2. The greatest similarity among pathogenic species existed between *A. fumigatus* and *A. niger*, with 52 nucleotide base differences (91.7% similarity), whereas *A. ustus* and *A. terreus* showed the greatest diversity, with differences at 128 nucleotide positions (79.3% similarity). *Aspergillus* ITS sequences generated in our laboratory from ATCC strains were compared with all *Aspergillus* sequences available in GenBank following the deposition of

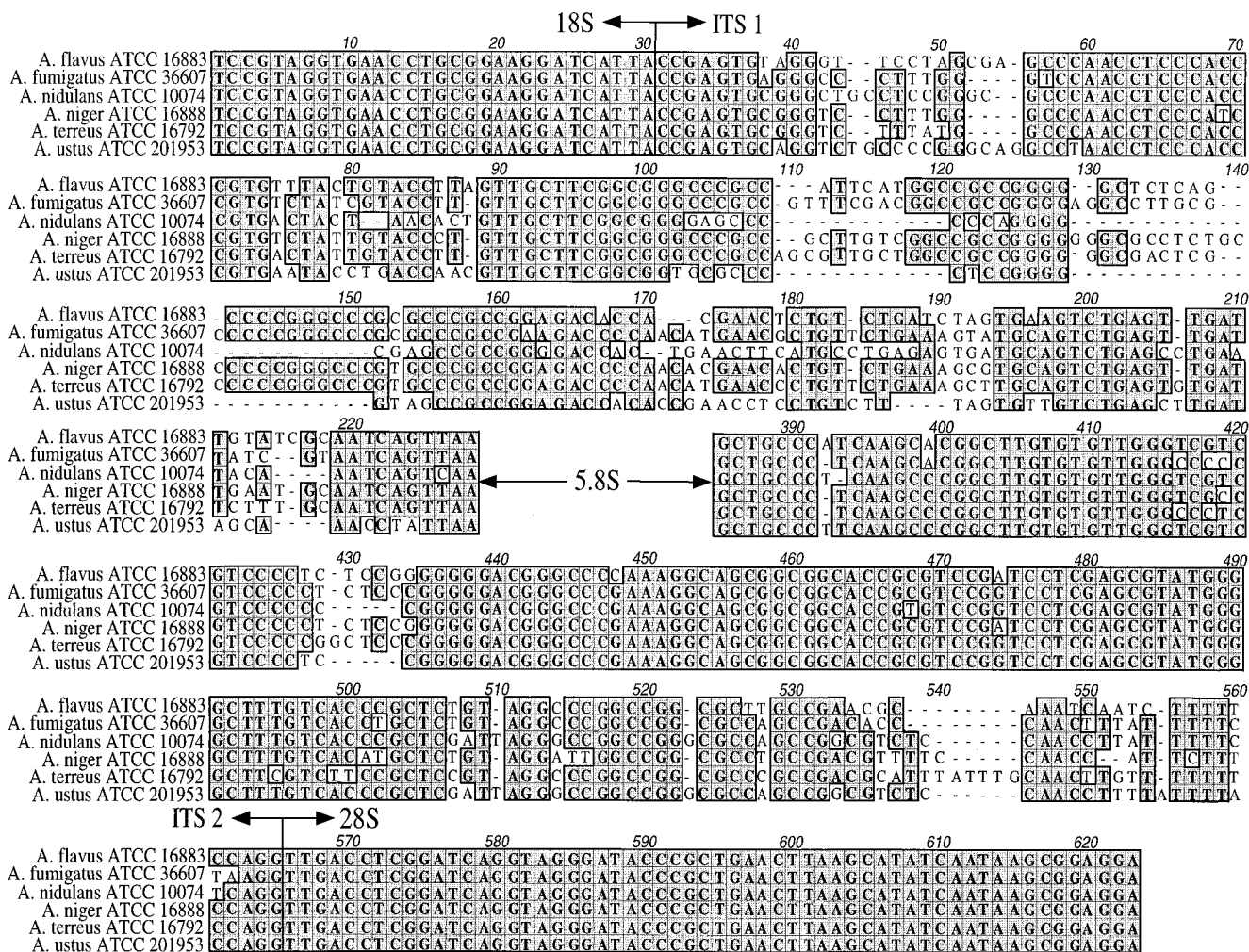


FIG. 1. Nucleotide sequence alignment of *A. flavus* (ATCC 16883), *A. fumigatus* (ATCC 36607), *A. nidulans* (ATCC 10074), *A. niger* (ATCC 16888), *A. terreus* (ATCC 16792), and *A. ustus* (ATCC 201953). The alignment consists of the 3' end of the 18S ribosomal DNA (rDNA) gene (which contains the ITS 1 primer site), the complete ITS 1 region, the complete ITS 2 region, and the 5' end of the 28S rDNA gene (which contains the ITS 4 primer site). The highly conserved 5.8S rDNA gene sequence has been omitted.

sequences listed in Table 3. For *A. flavus*, *A. fumigatus*, and *A. terreus*, the interspecies sequence similarity with all *Aspergillus* GenBank sequences (referenced and nonreferenced) was found to be less than 99%. A sequence similarity of 99% between *A. nidulans* (accepted into GenBank as *E. nidulans*) and *Emericella quadrilineata* was observed. A sequence similarity of 99% was also found among species within the *A. niger* aggregate including *A. phoenicis* and *A. tubigenis*.

**Sequence similarity of clinical isolates and reference strains of the same species.** The results of comparisons between clinical isolates and referenced strain sequences of the same *Aspergillus* species are shown in Table 3. The greatest intraspecies variation was seen among isolates of *A. fumigatus* and isolates of *A. niger*. For both species, five nucleotide base differences between the sequences of clinical isolates and that of the referenced strain existed. Considering the length of the ITS region amplified, the overall sequence similarity between the referenced *Aspergillus* strains and clinical isolates of the same species was greater than 99%.

**Sequence comparisons with other true pathogenic and opportunistic fungi.** To evaluate the utility of ITS sequences for identification of true pathogenic and opportunistic fungi, the

ITS 1–5.8S–ITS 2 region sequences of 12 different genera known to cause infection in humans were determined in our laboratory and compared to sequences from the six medically important aspergilli. The results obtained with *A. fumigatus* are shown in Table 4. Sequence similarities between *A. fumigatus* and the listed genera ranged from 50.2 to 89.6%, with *Penicillium* species showing the greatest sequence similarity. BLAST search comparisons between the other medically important *Aspergillus* species and all opportunistic fungi available in the GenBank database were also made (data not shown). The ITS 1 and 2 sequences of the referenced *Aspergillus* species differed from those of the other fungal genera by at least 1%, with one exception: *A. niger* ITS sequences had 99% sequence similarity with those of *Arthrobotrys* species and *Glaciocladium cibotii*. As expected, the referenced *A. niger* sequence was listed first in the bit score rank listing. To further test the system, the sequences of clinical isolates of *A. niger* were compared using an ungapped BLAST search of the GenBank database. In each case, the clinical isolate was distinguished from *Arthrobotrys* species and *G. cibotii* on the basis of bit score.

**Clinical validation of ITS sequence analysis.** To determine the utility of the ITS sequence for accurate identification of

TABLE 2. Matrix of ITS 1–5.8S–ITS 2 similarities for referenced *Aspergillus* species

Strain	% Similarity with strain:					
	<i>A. flavus</i> ATCC 16883	<i>A. fumigatus</i> ATCC 36607	<i>A. nidulans</i> ATCC 10074	<i>A. niger</i> ATCC 16888	<i>A. terreus</i> ATCC 16792	sp. <i>A. ustus</i> ATCC 201953
<i>A. flavus</i> ATCC 16883						
<i>A. fumigatus</i> ATCC 36607	87.6					
<i>A. nidulans</i> ATCC 10074 <sup>a</sup>	81.5	84.3				
<i>A. niger</i> ATCC 16888	89.6	91.7	84.0			
<i>A. terreus</i> ATCC 16792	87.0	91.1	83.0	90.6		
<i>A. ustus</i> ATCC 201953	82.7	80.7	91.4	80.5	79.3	

<sup>a</sup> Accepted into GenBank as *E. nidulans*.

*Aspergillus* species, a blind comparison using 11 morphologically confirmed *Aspergillus* clinical isolates was made. Following incubation of the culture plate for 24 h at 30°C and direct sequencing of PCR amplicons, ITS sequences were used in an ungapped BLAST search of the GenBank database. Identification of the unknown sequences was made using the highest bit score of listed species. By this method, each of the coded specimens was identified correctly as to the *Aspergillus* species. All of the identifications were made in less than 48 h after receipt of the blind culture plate.

## DISCUSSION

The increasing frequency of invasive fungal infection and the high mortality associated with disseminated fungal disease have highlighted the need for rapid identification of infectious molds from clinical samples. The number of cases of IA found at autopsy has increased 14-fold since 1978 (8). Early recognition and treatment of patients with invasive fungal infection are crucial, as the progression of invasive disease from detection to death is typically less than 14 days (4, 25). The present work was based on the premise that identification of *Aspergillus* at the species level will have clinical importance in the future. Currently, physicians rely on clinical findings and administer AmB empirically to immunosuppressed patients with sign and symptoms consistent with a fungal infection. However, the resistance of certain *Aspergillus* species to antifungal agents complicates empiric treatment for invasive disease (4, 14, 16). The effectiveness of AmB varies significantly depending on the species of *Aspergillus*, with over 95% of *A. terreus* isolates reported as resistant (10, 17, 22). Susceptibility testing has revealed a wide range of AmB MICs, from 0.5 µg/ml for *A. niger* and *A. fumigatus* to 16 µg/ml for *A. flavus* and *A. nidulans*. Thus, rapid diagnosis and recognition of the species causing infection and treatment with the most active antifungal therapy may be important to reducing the mortality of immunosuppressed patients with IA.

The detection of *Aspergillus* DNA from blood, serum, bronchoalveolar lavage fluid, and tissue has been accomplished by using the 18S rRNA gene as the target (6, 12, 24, 28). Einsele et al. detected *Aspergillus* DNA from blood approximately 4 days prior to the appearance of pulmonary infiltrates consistent with fungi by computed tomography scan in patients with presumed aspergillosis (6). While their report detailed the

TABLE 3. Numbers of nucleotide differences in ITS 1–5.8S–ITS 2 within a single species

Strain and accession no.	No. of nucleotide base differences in:			% Similarity
	ITS 1	ITS 2	ITS 1–5.8S–ITS 2	
<i>A. flavus</i> ATCC 16883				
IMI 210	0	1	1	99.8
AB008414	0	1	1	99.8
AB008415	0	2	2	99.7
AB008416	0	0	0	100.0
AF027863	0	0	0	100.0
AF078893	0	1	1	99.8
AF078894	0	0	0	100.0
L76747	4	0	4	99.3
<i>A. fumigatus</i> ATCC 36607				
IMI 196	2	2	5	99.2
AF078889	2	0	2	99.7
AF078890	1	0	1	99.8
AF078891	1	0	1	99.8
AF078892	1	0	1	99.8
<i>A. nidulans</i> ATCC 10074				
IMI 231	2	2	4	99.3
L76746 <sup>a</sup>	0	0	0	100.0
U03521 <sup>a</sup>	NA <sup>c</sup>	2	2	99.6
<i>A. niger</i> ATCC 16888				
IMI 026	0	0	0	100.0
AF078895	0	0	0	100.0
AJ223852	4	1	5	99.2
L76748	0	0	0	100.0
U65306	0	0	0	100.0
<i>A. terreus</i> ATCC 16792				
IMI 203	4	0	4	99.3
AF078896	0	0	0	100.0
AF078897	0	0	0	100.0
AJ001334	0	0	0	100.0
AJ001335	0	0	0	100.0
AJ001338	0	0	0	100.0
AJ001368	0	1	1	99.8
L76774	0	0	0	100.0
U93684	0	3	3	99.5
<i>A. ustus</i> UAMH 9479				
IMI 192 <sup>b</sup>	0	1	1	99.8

<sup>a</sup> Deposited into GenBank as *E. nidulans*.

<sup>b</sup> Deposited into the ATCC as ATCC 201953.

<sup>c</sup> NA, not available.

shortened time span to positive identification of *Aspergillus* from patient material, it was not possible to identify *Aspergillus* at the species level using the 18S rRNA gene (12). Additionally, the identification of aspergilli by PCR in some patient specimens, such as bronchoalveolar lavage fluid, does not always indicate invasive disease, and therefore the use of PCR for detection of fungi in specimens from potentially colonized sites may be limited.

The ITS regions have been used as targets for phylogenetic analysis because they generally display sequence variation between species, but only minor variation within strains of the same species (11, 13, 20, 21). Shin et al. have described a fluorescent DNA probe assay using the ITS 2 region for the identification of *Candida* species (19). Their approach was reliable for the detection of *Candida*, as 95.1% of *Candida* isolates tested were identified to the species level with 100%

TABLE 4. Nucleotide base differences in ITS 1–5.8S–ITS 2 between *A. fumigatus* and other medically important fungal genera

Species and accession no.	No. of nucleotide base differences in:			% Similarity <sup>a</sup>
	ITS 1	ITS 2	ITS 1–5.8S–ITS 2	
<i>Ajellomyces capsulatus</i>				
AF038353	93	45	143	76.6
AF156892 <sup>b</sup>	86	59	150	76.7
<i>Ajellomyces dermatitidis</i>				
AF038355	93	66	163	74.1
<i>Candida albicans</i>				
AF217609 <sup>b</sup>	108	98	221	65.0
L28817	97	98	211	64.8
<i>Cladophialophora bantiana</i>				
AF131079 <sup>b</sup>	82	111	202	68.1
<i>Cryptococcus neoformans</i>				
AF162916 <sup>b</sup>	99	123	237	59.1
L14067	53	126	193	59.4
<i>Cylindrocarpon lichenicola</i>				
AF133845 <sup>b</sup>	102	79	185	69.2
<i>Fusarium oxysporum</i>				
AF132799	85	91	180	62.3
<i>Fusarium solanii</i>				
U38558	100	92	202	66.7
<i>Fusarium</i> spp.				
IMI 183	99	89	197	67.0
<i>Gymnascella hyalinospora</i>				
AF129854 <sup>b</sup>	87	57	149	76.3
<i>Penicillium capsulatum</i>				
AF033429	44	23	70	88.0
<i>Penicillium glabrum</i>				
AF033407	39	22	62	89.6
<i>Penicillium marnefeii</i>				
ATCC 18224 <sup>c</sup>	60	57	124	79.1
L37406	57	54	116	79.5
<i>Phialophora verrucosa</i>				
AF050281	78	104	196	67.5
<i>Pseudallescheria boydii</i>				
AF022486	106	131	248	55.6
AF181558 <sup>b</sup>	109	132	252	55.4
<i>Saccharomyces cerevisiae</i>				
Z95929	144	146	302	50.2

<sup>a</sup> Compared to *A. fumigatus* ATCC 36607.

<sup>b</sup> Sequence deposited into GenBank as part of this study.

<sup>c</sup> Reference strain sequenced but not deposited into GenBank.

specificity. In addition, species level identification of six medically relevant *Trichosporon* isolates was achieved by using a highly variable 12-bp region within the ITS 1 and 2 regions (21). Gaskell et al. investigated sequence variation in ITS regions to distinguish *Aspergillus* from other allergenic molds (7). They found little variation between *Aspergillus* and *Penicillium* within the ITS 2 region but concluded that the ITS 1 region may be sufficient for identification. Although *Penicillium capsulatum* and *Penicillium glabrum* exhibited the highest sequence similarity to *Aspergillus* species in our study, the presence of a 10-bp sequence variation within the ITS 2 region allowed these species to be readily distinguished. We therefore concluded that both the ITS 1 and 2 regions were necessary for species level identification. A limited number of strains were available for some *Aspergillus* species, particularly *A. ustus*, which was not previously listed in the GenBank database. Although incomplete, the sequences of GenBank nonreferenced strains showed little difference from those of ATCC referenced strains.

Variation in ITS 2 amplicon size was used by Turenne et al. to identify clinically important fungi using CE for separation and identification (23). They tested 56 fungi and were able to identify 48 at the species level. Similar to our results, they found only a two-nucleotide base difference when comparing the lengths of *A. flavus*, *A. niger*, and *Fusarium solani* ITS amplicons. This suggested that amplicon lengths may not be sufficiently different to distinguish species. We also found *A. niger* and *A. terreus* amplicons to be similar in length. The resolution of CE is approximately two nucleotides for amplicons greater than 250 bases in length. It is not clear whether the technical limitations of CE make it a reliable method for species level identification of *Aspergillus*.

The comparison of ITS 1–5.8S–ITS 2 region sequences among referenced and clinical isolates of six *Aspergillus* species revealed several areas of sequence variation. The inclusion of the 5.8S rRNA gene sequence had minimal impact on the overall comparison since there is little interspecies variation in this region. In our study, the intraspecies variation among clinical and pathogenic referenced *Aspergillus* strains was less than 1%. This is consistent with the phylogenetic study by Sugita et al. of the *Trichosporon* species, where less than 1% of nucleotide bases were different among various strains of the same species (21).

Gaskell et al. have previously shown that *Alternaria*, *Penicillium*, *Cladosporium*, and *Aspergillus* could be differentiated at the genus level on the basis of ITS sequence analysis (7). The question remained, however, whether ITS sequences could be used to identify any fungus that may be recovered clinically, including those that may be environmental contaminants. In our study, a BLAST search of all GenBank sequences was conducted using the six referenced *Aspergillus* species ITS sequences. Sequence similarities of less than 89.6% were seen when comparing the ITS region sequences of *A. fumigatus* to those of other genera, including opportunistic fungi and true pathogenic fungi listed in Table 4. This search also identified two species, *A. nidulans* and *A. niger*, that had sequence similarities of 99% with other opportunistic fungi.

*A. nidulans* (deposited in GenBank as *E. nidulans*) ITS sequences had 99% sequence similarity with those of *E. quadrilineata*. However, *E. quadrilineata* has not been reported as a cause of invasive disease in humans. *A. niger* ITS sequences were found to be similar to those of nonreferenced isolates of *A. phoenicis*, *A. tubigenensis*, *Arthrotrichum* species, and *G. cibotii*. The *A. niger* aggregate includes two subgroups and at least 14 species, including *A. phoenicis* and *A. tubigenensis*, that are morphologically indistinguishable. By contrast, *Gliocladium* and *Arthrotrichum* species have morphological features distinct from those of *A. niger*. Again, none of these species have been associated with invasive disease, and their medical importance is unknown (18). Additional studies to confirm the ITS sequences of referenced isolates of these infrequently encountered fungal species are in progress. Overall, the present results showed that ITS sequence analysis can be used to exclude fungal genera which may be considered in the differential diagnosis of a patient with invasive mycosis. However, the sequence similarity of 99% with some genera and species indicated that the BLAST bit score would be needed to identify clinical isolates of *Aspergillus* to the species level. A correct identification of clinical isolates of *A. niger* and *A. nidulans* was made using the highest bit score of listed species from the BLAST search. This demonstrated that ITS 1 and 2 sequence analysis can be used for recognition of many fungal genera, including those that do not typically cause invasive disease such as airborne allergenic fungi.

Our studies showed that it was not necessary to clone the

PCR products to obtain an accurate reading of the sequence. The elimination of this step allowed for direct automated sequencing of PCR products and significantly reduced the amount of time involved in obtaining a result. The ability to sample small (approximately 2-mm<sup>2</sup>) portions of the culture contributed significantly to rapid identification. Colonies of this size generally cannot be used for morphological identification, and in most cases the specimen must be incubated for 5 days or longer. The ability to rapidly and accurately identify *Aspergillus* species from blind samples, with results available within 48 h, confirmed the value of this approach. Several issues may affect the time required to obtain a result, including the availability of a dedicated sequencer. The need to repeat the sequencing procedure due to gel compression or contamination may also delay the process. Although automated sequencing and analysis provided accurate discrimination of *Aspergillus* from other fungi, a probe-based DNA hybridization approach for other organisms has been described and may be more cost effective in the future (6, 19).

Identification of medically important *Aspergillus* species from short-term culture using nucleic acid sequence analysis of the ITS 1 and 2 regions in combination with a BLAST bit score is a reliable and efficient method that provides earlier identification than standard culture methods. The identification of rarely encountered opportunistic organisms following sequence analysis should prompt a review of the sequence data and correlation with clinical findings. Investigations are in progress to determine whether the method has utility for direct identification of fungi in tissue sections where histologic evidence of a fungus exists. Additional studies are needed to demonstrate whether identification of *Aspergillus* at the species level will improve patient outcome through the selection of more-effective antifungal therapy.

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