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Assessment of Ribosomal Large-Subunit D1-D2, Internal Transcribed Spacer 1, and Internal Transcribed Spacer 2 Regions as Targets for Molecular Identification of Medically Important *Aspergillus* Species

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Molecular approaches are now being developed to provide a more rapid and objective identification of fungi compared to traditional phenotypic methods. Ribosomal targets, especially the large-subunit RNA gene (D1-D2 region) and internal transcribed spacers 1 and 2 (ITS1 and ITS2 regions), have shown particular promise for the molecular identification of some fungi. We therefore conducted an assessment of these regions for the identification of 13 medically important *Aspergillus* species: *Aspergillus candidus*, *Aspergillus* (*Eurotium*) *chevalieri*, *Aspergillus* (*Fennellia*) *flavipes*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus granulosus*, *Aspergillus* (*Emericella*) *nidulans*, *Aspergillus niger*, *Aspergillus restrictus*, *Aspergillus sydowii*, *Aspergillus terreus*, *Aspergillus ustus*, and *Aspergillus versicolor*. The length of ribosomal regions could not be reliably used to differentiate among all *Aspergillus* species examined. DNA alignment and pairwise nucleotide comparisons demonstrated 91.9 to 99.6% interspecies sequence identities in the D1-D2 region, 57.4 to 98.1% in the ITS1 region, and 75.6 to 98.3% in the ITS2 region. Comparative analysis using GenBank reference data showed that 10 of the 13 species examined exhibited a ≤1-nucleotide divergence in the D1-D2 region from closely related but different species. In contrast, only 5 of the species examined exhibited a ≤1-nucleotide divergence from sibling species in their ITS1 or ITS2 sequences. Although the GenBank database currently lacks ITS sequence entries for some species, and major improvement in the quality and accuracy of GenBank entries is needed, current identification of medically important *Aspergillus* species using GenBank reference data seems more reliable using ITS query sequences than D1-D2 sequences, especially for the identification of closely related species.

Aspergillus species are an increasingly important cause of invasive fungal infections in immunocompromised patients (31, 59). Unfortunately, there are few specific clinical signs of invasive aspergillosis and current methods for laboratory diagnosis are less than ideal, particularly in the early stages of the disease (8, 49). Given the recent reports of reduced antifungal drug susceptibilities among some *Aspergillus* species (21, 26, 50), the timely and accurate identification of aspergilli to the species level has become especially important (10). Species identification is also important for epidemiological purposes and as a guide to clinical management (29, 47, 48).

The current laboratory identification of *Aspergillus* species is based on macroscopic colonial and microscopic morphological characteristics (7, 20, 45). Over 180 different species in at least 16 subgeneric groups or sections can be distinguished (35, 37, 38), including approximately 30 species which are recognized as opportunistic pathogens of humans (7). Many clinical laboratories use traditional phenotypic methods of identification and can differentiate only the more common *Aspergillus* species; the delineation of less common species must be referred to specialist laboratories. In addition, species identification by traditional phenotypic methods may require several weeks to accomplish, and in a small number of cases, isolates may not conidiate, obstructing species identification (20, 38). Therefore, rapid molecular approaches are now being developed to replace the need for culture by detecting and identifying *Aspergillus* species DNA directly from clinical materials.

A number of targets for the molecular identification of aspergilli have been investigated including the mitochondrial cytochrome *b* gene (46, 57, 62), a putative aflatoxin pathway regulatory gene (*aflR*) (4), the DNA topoisomerase II gene (*TOP2*) (19), the β-tubulin gene (11), and various rRNA gene regions (18). The most promising targets to date have been the 5′ end of the large-subunit rRNA gene (D1-D2 region) (35) and the internal transcribed spacer 1 and 2 (ITS1 and ITS2) regions between the small- and large-subunit rRNA genes (18, 60). The use of DNA sequence diversity in the ribosomal regions as an aid to species identification has been exploited using PCR amplification of targets followed by either fragment length analysis (1, 16, 22, 39, 52, 56), DNA probe hybridization (15, 30, 32, 61), or DNA sequence analysis (3, 17, 35, 43, 55, 61). In general, DNA-based approaches were found to provide more reliable and faster species identifications than culture-based methods. Nonetheless, some diagnostic problems were encountered, resulting from similar amplicon or fragment lengths for different taxa (16, 39, 52), unexpected cross-hybridization results (9, 16, 30, 32, 42, 61), and ambiguous sequencing-based identifications because of significant intraspecies heterogeneity (44, 53, 54) or virtually identical sequences for apparently distinct organisms (14, 17, 35, 43). The validation of

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test specificity was further compromised by the absence of a comprehensive test strain panel; most studies did not include type strains or authenticated culture collection reference strains of the target species or of closely related species in their examinations. Finally, although the D1-D2 and the ITS1 and ITS2 regions have been analyzed separately for some species of *Aspergillus*, a systematic evaluation and comparison of sequences from all three ribosomal regions for their usefulness in the identification and differentiation of the most medically important *Aspergillus* species has not been published to date.

Therefore, the present investigation assessed the utility of the ribosomal D1-D2, ITS1, and ITS2 regions as targets for the molecular identification of 13 potentially invasive *Aspergillus* species (*Aspergillus candidus*, *Aspergillus chevalieri*, *Aspergillus flavipes*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus granulosus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus restrictus*, *Aspergillus sydowii*, *Aspergillus terreus*, *Aspergillus ustus*, and *Aspergillus versicolor*). We acquired DNA sequence information concerning all three ribosomal regions for each of the above species and used this information to conduct DNA sequence alignments, pairwise nucleotide sequence analyses, comparative GenBank database searches, and an examination for sequence length polymorphisms among *Aspergillus* species.

MATERIALS AND METHODS

Microorganisms. A total of 36 reference strains, including both type strains and authenticated culture collection strains for the following medically important *Aspergillus* species, were investigated: *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* (Table 1). Isolates were grown on Czapek-Dox agar (Difco Laboratories, Detroit, Mich.) for up to 14 days at 25°C and up to 1 week at 37°C to confirm their purity and species identity based on traditional criteria (38), and to generate fungal growth for DNA extraction.

DNA extraction. Fungal biomass, scraped from the surface of one agar plate, was transferred into a precooled (−20°C) sterile ceramic mortar, overlaid with liquid nitrogen, and carefully ground with a sterile ceramic pestle into a fine powder. The powder was suspended in 2 ml of buffer G-2 (genomic DNA buffer set; Qiagen, Valencia, Calif.) containing RNase (200 μg/ml; Sigma Chemical Company, St. Louis, Mo.) and transferred into a clean test tube. Following addition of 45 μl of proteinase K solution (20-mg/ml stock solution; Sigma), the suspension was incubated with intermittent agitation at 55°C for 3 h. The crude extract was centrifuged at 21,500 × *g* for 10 min, and the supernatant was transferred into a clean test tube. DNA was then purified using Genomic-tip 20/G columns (Qiagen) according to the manufacturer's instructions. The eluted DNA was supplemented with 2.5 μl of glycogen solution (20 mg/ml; Gentra Systems, Minneapolis, Minn.), precipitated by standard methods using isopropanol and ethanol (41), and resuspended in 60 μl of DNA rehydration buffer (PureGene kit, Gentra Systems). DNA was then stored at −20°C until used.

PCR amplification of the D1-D2 region of the large-subunit (28S) rRNA gene. Seminested PCR using broad-range primer pairs ITS1 (5′ TCC GTA GGT GAA CCT GCG G) and D2R (5′ TTG GTC CGT GTT TCA AGA CG) followed by D1 (5′ GCA TAT CAA TAA GCG GAG GA) and D2R generated the D1-D2 region amplicons for sequencing (36, 60). PCR amplification was conducted in a GeneAmp model 9700 thermal cycler (Perkin Elmer Applied Biosystems, Foster City, Calif.), and PCR components were obtained from Roche Molecular Biochemicals, Indianapolis, Ind. The PCR mix contained 0.2 mM concentrations of each deoxynucleoside triphosphate, 1.25 U of *Taq* DNA polymerase, 0.2 μM concentrations of each primer, 2 μl of a 1:50 dilution of the original DNA extract (>5 ng of DNA for the initial PCR or 2 μl of undiluted amplicon from the initial PCR when products were reamplified in the seminested PCR), and the appropriate amount of PCR buffer (final concentration, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl) to bring the final volume to 50 μl. One sample without DNA template was always included as a negative control. Amplification with the primer pair ITS1 and D2R was performed using a denaturation step of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 51°C for 30 s, and 72°C for 150 s. A final extension step at 72°C for 10 min was then used. Reamplification with primer pair D1 and D2R for the seminested PCR amplification included denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 90 s. A final extension step at 72°C for 7 min was then employed.

PCR amplification of the ribosomal ITS1 and ITS2 regions. The broad-range primer pairs ITS5 (5′ GGA AGT AAA AGT CGT AAC AAG G) and ITS2 (5′ GCT GCG TTC TTC ATC GAT GC) or ITS3 (5′ GCA TCG ATG AAG AAC GCA GC) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC) were used to amplify ribosomal internal transcribed spacer regions 1 and 2, respectively (60). Some samples required seminested PCR amplification with primer pair ITS5 and ITS4 followed by ITS5 and ITS2 or ITS3 and ITS4 to generate sufficient quantities of PCR amplicon for DNA sequencing. Amplifications and reamplifications were performed as described above, except for the primer pair used and minor differences in the thermal cycling conditions employed: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. A final extension step at 72°C for 5 min was then conducted.

DNA sequencing. All PCR products were purified before DNA sequence analysis using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Purified amplicons were then sequenced on both strands using the same primers as described above. BigDye terminator cycle sequencing Ready Reaction kits (Perkin Elmer Applied Biosystems) were employed as recommended by the manufacturer. All cycle sequencing reactions were performed on a GeneAmp model 9700 thermal cycler using an initial denaturation at 96°C for 5 s, followed by 30 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Products were purified using a Dye-Ex spin kit (Qiagen), dried in a vacuum centrifuge, and resuspended in either template suppression reagent (Perkin Elmer Applied Biosystems; D1/D2R products) or formamide (Perkin Elmer Applied Biosystems; ITS products). Products were then analyzed on an automated capillary DNA sequencer (ABI Prism 310 genetic analyzer; Perkin Elmer Applied Biosystems) according to the manufacturer's directions. Comparative sequence analysis and GenBank searches were assisted by the Genetics Computer Group software package (FASTA, BESTFIT, STRETCHER, and PILEUP algorithms; University of Wisconsin, Madison), the Clustal W alignment program (51), and the nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST) algorithm (blastn) (2, 63). Calculation of pairwise sequence identity was restricted to full-length reference data of *Aspergillus* species currently sanctioned by the International Commission of *Penicillium* and *Aspergillus* (37). Authoritative *Aspergillus* reference sequences were taken from the work of Peterson (35), Haugland et al. (15), and Pazoutova et al. (34).

Nucleotide sequence accession numbers. The *Aspergillus* large-subunit D1-D2, ITS1, and ITS2 ribosomal regions determined in this study were deposited in GenBank and assigned the accession numbers listed in Table 1. The species designations are in compliance with currently sanctioned names published by the International Commission of *Penicillium* and *Aspergillus* (37).

RESULTS

Traditional and molecular classification of the *Aspergillus* strains used in this study. Type strains and authenticated culture collection reference strains, representing 13 of the most common and clinically relevant *Aspergillus* species, were used for DNA sequence analysis of the D1-D2, ITS1, and ITS2 ribosomal regions. Species designations were based on culture collection information provided with the isolates and on phenotypic reconfirmation according to Raper and Fennell (38) at the Centers for Disease Control and Prevention, Atlanta, Georgia. Strain designations and the corresponding GenBank accession numbers for our DNA sequences are listed in Table 1. Strains marked with a superscript "T" represent type strains according to information provided on the website of the Agricultural Research Service Culture Collection (http://nrrl.ncaur.usda.gov); strains marked with a superscript "MT" represent non-type strains with sequences that perfectly matched the type strain reference data in GenBank (15, 35) in all ribosomal regions investigated. For the purposes of this study, strains which exhibited minor sequence divergence compared to the corresponding type strain sequences, as determined in this study, or compared to the corresponding type strain reference sequences available in GenBank (15, 35) are designated

TABLE 1. Ribosomal *Aspergillus* sequences determined in this study

Species and sequevar[a]	Strain[c]	GenBank accession no. (length in nucleotides)[b] per target			Total size of ITS region[d]
		D1-D2	ITS1	ITS2	
A. candidus	NRRL 303[T]	AF454148	AF453881 (180)	AF454098 (168)	**505**
	NRRL 312[MT]	AF454149	AF453882 (180)	AF454099 (168)	505
A. (*Eurotium*) *chevalieri*	ATCC 16443[T]	AF454150	AF453883 (142)	AF454100 (167)	**466**
	ATCC 24546[MT]	AF454151	AF453884 (142)	AF454101 (167)	466
A. (*Fennellia*) *flavipes* I	ATCC 11013	AF454154[e]	AF453886 (185)[e]	AF454104 (163)[e]	505
A. (*Fennellia*) *flavipes* II	ATCC 16805	AF454155[e]	AF453887 (184)[e]	AF454105 (160)[e]	501
A. (*Fennellia*) *flavipes* III	ATCC 24487[T]	AF454156	AF453888 (185)	AF454106 (162)	**504**
A. flavus	ATCC 11497[MT]	AF454158	AF453890 (181)	AF454108 (169)	**507**
	ATCC 34896[MT]	AF454159	AF453891 (181)	AF454109 (169)	507
	ATCC 44310[MT]	AF454160	AF453892 (181)	AF454110 (169)	507
	ATCC 64025[MT]	AF454161	AF453893 (181)	AF454111 (169)	507
A. fumigatus	ATCC 1022[T]	AY660917	AY660920 (184)	AY660923 (168)	**509**
	ATCC 16903[MT]	AF454163	AF453895 (184)	AF454113 (168)	509
	CDC B2570[MT]	AF454164	AF453896 (184)	AF454114 (168)	509
A. granulosus	CBS 119.58[MT]	AF454165	AF453897 (156)	AF454115 (171)	484
	NRRL 1932[T]	AF454166	AF453898 (156)	AF454116 (171)	**484**
A. (*Emericella*) *nidulans* I	ATCC 10074[T]	AY660918	AY660921 (153)	AY660924 (168)	**478**
A. (*Emericella*) *nidulans* II	ATCC 16855	AF454167	AF453899 (153)[e]	AF454117 (168)	478
A. (*Emericella*) *nidulans* III	CDC B6597	AF454192[e]	AF453924 (154)[e]	AF454141 (169)[e]	480
A. niger I	ATCC 1015[MT]	AF454169	AF453901 (185)	AF454118 (169)	**511**
	ATCC 64028[MT]	AF454171	AF453903 (185)	AF454120 (169)	511
A. niger II	ATCC 16404	AF454170	AF453902 (185)[e]	AF454119 (170)[e]	512
A. restrictus	NRRL 148[MT]	AF454175	AF453907 (181)	AF454124 (172)	**510**
	NRRL 151[MT]	AF454176	AF453908 (181)	AF454125 (172)	510
A. sydowii I	NRRL 250	AF454177[e]	AF453909 (155)[e]	AF454126 (168)[e]	480
	NRRL 4768	AF454178[e]	AF453910 (155)[e]	AF454127 (168)[e]	480
A. sydowii II	NRRL 254[T]	AY660919	AY660922 (155)	AY660925 (168)	**480**
A. terreus	ATCC 1012[T]	AF454183	AF453915 (186)	AF454132 (177)	**520**
	ATCC 1002[MT]	AF454184	AF453916 (186)	AF454133 (177)	520
	ATCC 7860[MT]	AF454185	AF453917 (186)	AF454134 (177)	520
A. ustus I	ATCC 14417	AF454186[e]	AF453918 (155)[e]	AF454135 (170)[e]	482
A. ustus II	ATCC 16801	AF454187[e]	AF453919 (155)[e]	AF454136 (170)[e]	482
A. ustus III	NRRL 275[T]	AF454188	AF453920 (161)	AF454137 (172)	**490**
A. versicolor	ATCC 10072[MT]	AF454193	AF453925 (155)	AF454142 (167)	479
	NRRL 238[T]	AF454194	AF453926 (155)	AF454143 (167)	**479**
	NRRL 239[MT]	AF454195	AF453927 (155)	AF454144 (167)	479

[a] Roman numerals placed after a species name represent different ribosomal sequevars of the same species (minor divergence in one or more ribosomal regions compared to the corresponding type strain sequences, as determined in this study, or compared to the corresponding type strain reference sequences available in GenBank; see Results).

[b] Sequences containing the D1-D2 region were 542 nt in length for all species except *A. nidulans* (543 nt).

[c] A superscript "T" designates type strains according to information provided on the website of the Agricultural Research Service Culture Collection (http://nrrl.ncaur.usda.gov); "MT" designates non-type strains that gave sequence information that perfectly matched the type strain reference data (15, 35) in all ribosomal regions investigated in this study. ATCC, American Type Culture Collection, Manassas, Va; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; NRRL, National Regional Reference Laboratory, Peoria, Ill.

[d] Length of complete ITS1-ITS2 region (including the intervening 5.8S rRNA gene that exhibited a conserved length of 157 nt in all species investigated in this study). Boldface type indicates overall sequence length for type strain sequences or sequences matching type strain reference data (15).

[e] Sequences in these target regions did not match those for the corresponding type strain.

in Table 1 by roman numerals after the species name to represent different sequevars of the same species; ribosomal regions where divergence from the type strain sequences occurred were identified in Table 1 with a superscript "e."

Use of the D1-D2, ITS1, and ITS2 DNA sequence length to discriminate among *Aspergillus* species investigated in this study. The D1-D2 sequencing results for all *Aspergillus* species studied showed identical overall lengths for all species and strains investigated (i.e., 542 nucleotides [nt]) with the exception of *A. nidulans* (total length, 543 nt [Table 1]). In contrast, the ITS1 region ranged in overall length from 142 nt (*A. chevalieri*) to 186 nt (*A. terreus*), and sequence length was more

TABLE 2. Pairwise sequence comparison in D1-D2 regions between medically important *Aspergillus* species investigated in this study

Species no.	Species and strain[b]	% Sequence identity with species no.[a]:												
		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*A. fumigatus* ATCC 1022	100												
2	*A. flavus* ATCC 11497	94.3	100											
3	*A. niger* ATCC 1015	94.6	96.7	100										
4	*A. terreus* ATCC 1012	95.6	95.2	95.8	100									
5	*A. flavipes* ATCC 24487	95.9	96.5	95.8	96.5	100								
6	*A. candidus* NRRL 303	95.6	96.9	96.1	95.6	96.7	100							
7	*A. restrictus* NRRL 148	93.4	92.8	93.4	93.5	93.4	93.2	100						
8	*A. chevalieri* ATCC 16443	94.5	94.5	94.5	94.6	95.0	94.6	95.8	100					
9	*A. granulosus* NRRL 1932	93.9	94.5	95.2	93.4	95.0	93.9	93.0	94.3	100				
10	*A. ustus* NRRL 275	93.5	94.1	94.8	93.0	94.6	93.5	92.6	93.9	99.6	100			
11	*A. sydowii* NRRL 254	93.2	93.5	94.5	93.0	94.1	93.2	91.9	93.4	98.3	98.0	100		
12	*A. versicolor* NRRL 238	93.4	93.7	94.6	92.8	94.3	93.4	92.1	93.5	98.5	98.5	99.1	100	
13	*A. nidulans* ATCC 10074	94.5	94.7	95.4	93.6	94.8	94.1	92.8	93.4	98.7	98.7	97.4	97.6	100

[a] Values were calculated from pairwise alignments using sequence data determined in this study (for GenBank accession numbers, see Table 1) and the STRETCHER algorithm. Species numbers were assigned arbitrarily.
[b] Sequences were derived from type strains or from non-type strains (*A. flavus* ATCC 11497, *A. niger* ATCC 1015, and *A. restrictus* NRRL 148) that exhibited perfect sequence identity with corresponding type strain reference data (35).

divergent among *Aspergillus* species in the ITS1 region than in the D1-D2 region; i.e., in the ITS1 region, 5 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. In addition, the overall sequence length for the type strain of *A. ustus* (NRRL 275) not only was unique compared to other *Aspergillus* species but differed by 6 nt from its own sequevars (i.e., 161 nt versus 155 nt); these data suggest substantial divergence of these sequevars from the type strain. However, all of the 7 remaining species and the two divergent *A. ustus* sequevars (I and II) shared identical overall sequence length with at least one strain from each of the 13 species examined (e.g., *A. flavipes* and *A. niger*, 185 nt [Table 1]). Identical or similar overall sequence lengths were observed for *A. candidus*, *A. flavus*, and *A. restrictus* (180 to 181 nt), *A. flavipes*, *A. fumigatus*, *A. niger*, and *A. terreus* (184 to 186 nt), and *A. granulosus*, *A. nidulans*, *A. sydowii*, *A. ustus* (sequevars I and II), and *A. versicolor* (153 to 156 nt). In some instances, overall sequence length differences varied more within a given species than between species and ranged from 1 nt (*A. flavipes*, *A. nidulans*) to 6 nt (*A. ustus*) (Table 1).

In comparison, ITS2 regions varied in overall length from 160 nt (*A. flavipes*) to 177 nt (*A. terreus*) (Table 1). Overall sequence length was slightly less divergent among the *Aspergillus* species in the ITS2 than in the ITS1 region but was more divergent than in the D1-D2 region (Table 1). Three of the 13 species examined demonstrated a unique overall sequence length in the ITS2 region (*A. chevalieri*, *A. granulosus*, and *A. terreus*), and 5 shared identical overall sequence length with at least 1 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 to 169 nt) and among *A. granulosus*, *A. restrictus*, and *A. ustus* (170 to 172 nt). In some instances, similar to the results obtained in the ITS1 analysis, overall sequence length differences in the ITS2 region varied more within a given species than between species and ranged from 1 nt (e.g., *A. niger*) to 3 nt (*A. flavipes*) (Table 1).

Therefore, as noted for data generated using the D1-D2 and ITS1 regions, the overall sequence length of the ITS2 region alone could not be used reliably to differentiate among all 13 *Aspergillus* species examined. However, when data was combined for both the ITS1 and ITS2 regions, including or excluding the intervening, conserved, 5.8S rRNA gene, all 13 *Aspergillus* type strain sequences could be differentiated from one another (ITS1 plus ITS2 overall sequence length [Table 1]). The only overlaps in overall sequence length were between *A. candidus* (505 nt) and one non-type strain sequevar of *A. flavipes* (*A. flavipes* I; 505 nt) and between a non-type strain sequevar of *A. nidulans* (*A. nidulans* II, 480 nt) and *A. sydowii*.

Pairwise nucleotide sequence comparison of the D1-D2, ITS1, and ITS2 ribosomal regions among *Aspergillus* species investigated in this study. Table 2 shows the results of pairwise nucleotide sequence analysis of the D1-D2 region, and Tables 3 and 4 show the same analysis but for the ITS1 and ITS2 ribosomal regions. D1-D2 sequences were found to be highly conserved among the aspergilli (91.9 to 99.6% identity [Table 2]). The two species with the greatest similarity in nucleotide sequence were *A. ustus* and *A. granulosus* (99.6% identity); *A. sydowii* and *A. versicolor* sequences were also very similar and shared 99.1% sequence identity. Those species that were the most dissimilar in D1-D2 sequence were *A. sydowii* and *A. restrictus* (91.9% sequence identity). Phylogenetically related species, i.e., representatives of the *Aspergillus* subgenus *Nidulantes* (35), demonstrated more than 97% sequence identity: *A. granulosus*, 98.3 to 99.6% identity with *A. ustus*, *A. sydowii*, *A. versicolor*, and *A. nidulans*; *A. ustus*, 98.0 to 98.7% identity with *A. sydowii*, *A. versicolor*, and *A. nidulans*; and *A. sydowii*, 99.1% identity with *A. versicolor*. In addition, *A. sydowii* and *A. versicolor* shared 97.4 and 97.6% sequence identity, respectively, with *A. nidulans*.

In contrast to the D1-D2 region, pairwise nucleotide sequence analysis of the ITS1 region demonstrated significantly more variation in nucleotide sequences among the aspergilli

TABLE 3. Pairwise sequence comparison in ITS1 regions between medically important *Aspergillus* species investigated in this study

Species no.	Species and strain[b]	% Sequence identity with species no.[a]:												
		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*A. fumigatus* ATCC 1022	100												
2	*A. flavus* ATCC 11497	77.4	100											
3	*A. niger* ATCC 1015	85.0	79.5	100										
4	*A. terreus* ATCC 1012	84.0	78.0	87.8	100									
5	*A. flavipes* ATCC 24487	79.5	75.3	82.3	86.8	100								
6	*A. candidus* NRRL 303	84.0	82.3	82.4	79.8	78.7	100							
7	*A. restrictus* NRRL 148	83.1	75.0	83.0	80.4	77.4	78.9	100						
8	*A. chevalieri* ATCC 16443	63.8	60.0	62.6	60.4	57.8	61.5	71.6	100					
9	*A. granulosus* NRRL 1932	58.9	61.2	58.2	58.5	60.6	60.1	57.4	63.8	100				
10	*A. ustus* NRRL 275	59.0	60.4	59.2	60.5	58.7	58.7	59.2	60.1	92.5	100			
11	*A. sydowii* NRRL 254	62.6	61.8	60.7	59.5	59.5	63.2	61.7	68.5	73.9	72.6	100		
12	*A. versicolor* NRRL 238	62.6	61.3	62.3	60.5	60.1	62.3	61.7	69.8	73.3	70.7	98.1	100	
13	*A. nidulans* ATCC 10074	63.7	60.4	61.3	60.0	60.4	62.1	61.1	67.5	74.8	75.0	94.2	94.8	100

[a] Values were calculated from pairwise alignments using sequence data determined in this study (for GenBank accession numbers, see Table 1) and the STRETCHER algorithm. Species numbers were assigned arbitrarily.
[b] Sequences were derived from type strains or from non-type strains (*A. flavus* ATCC 11497, *A. niger* ATCC 1015, and *A. restrictus* NRRL 148) that exhibited perfect sequence identity with corresponding type strain reference data (15).

(Table 3). Sequence identities among the 13 *Aspergillus* species ranged from 57.4 to 98.1%. The two species with the greatest similarity in nucleotide sequence were *A. sydowii* and *A. versicolor* (98.1% identity) and those that were the most dissimilar were *A. granulosus* and *A. restrictus* (57.4% identity). Only four pairs of species, all belonging to the subgeneric *Nidulantes* group, demonstrated sequence identities greater than 92% (*A. ustus* versus *A. granulosus*, 92.5% identity; *A. versicolor* versus *A. sydowii*, 98.1% identity; *A. nidulans* versus *A. sydowii* or *A. versicolor*, 94.2 and 94.8% identity, respectively).

On the other hand, pairwise nucleotide sequence analysis of the ITS2 region demonstrated intermediate variation among *Aspergillus* species compared to the D1-D2 and ITS1 regions (Table 4). Sequence identities among the 13 *Aspergillus* species ranged from 75.6 to 98.3% identity. The two species with the greatest similarity in nucleotide sequence were *A. granulosus* and *A. ustus* (98.3% identity), agreeing with the D1-D2 similarity ranking; those that were the most dissimilar were *A. terreus* and *A. restrictus* (75.6% identity). Eight pairs of species, all belonging to the subgeneric *Nidulantes* group, demonstrated sequence identities between 93% and 98.3%: *A. granulosus* (93.6 to 98.3% identity with *A. versicolor*, *A. sydowii*, *A. nidulans*, and *A. ustus*), *A. ustus* (94.8% identity with *A. nidulans*), *A. sydowii* (95.9 and 97% identity with *A. nidulans* and *A. versicolor*, respectively), and *A. nidulans* (95.3% identity with *A. versicolor*).

Alignment of DNA sequences of the D1-D2, ITS1, and ITS2 ribosomal regions among *Aspergillus* species investigated in this study. DNA sequence alignments of the D1 and D2 regions and of the ITS1 and ITS2 regions were conducted to identify areas within each region which displayed the greatest diversity and which might best discriminate among the *Aspergillus* species examined. Although some interspecies sequence divergence was observed in the more conserved D1 region, the most significant sequence divergence among *Aspergillus* species occurred in the more variable D2 region (i.e., in the D1 region, 27 [19.7%] divergent sites occurred over 137 aligned positions; in the D2 region, 55 [26.3%] divergent sites

TABLE 4. Pairwise sequence comparison in ITS2 regions between medically important *Aspergillus* species investigated in this study

Species no.	Species and strain[b]	% Sequence identity with species no.[a]:												
		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*A. fumigatus* ATCC 1022	100												
2	*A. flavus* ATCC 11497	87.7	100											
3	*A. niger* ATCC 1015	87.1	87.2	100										
4	*A. terreus* ATCC 1012	85.9	83.3	83.6	100									
5	*A. flavipes* ATCC 24487	87.5	85.9	83.4	83.6	100								
6	*A. candidus* NRRL 303	89.4	86.0	85.9	84.2	86.3	100							
7	*A. restrictus* NRRL 148	81.5	80.0	79.5	75.6	78.0	83.3	100						
8	*A. chevalieri* ATCC 16443	80.1	80.9	75.9	76.4	76.6	82.5	83.2	100					
9	*A. granulosus* NRRL 1932	84.6	83.4	84.7	83.5	86.6	85.2	78.1	79.2	100				
10	*A. ustus* NRRL 275	85.3	83.6	84.1	82.3	85.5	84.2	78.8	78.7	98.3	100			
11	*A. sydowii* NRRL 254	85.1	83.8	85.5	82.2	87.0	86.1	78.5	78.8	94.2	91.9	100		
12	*A. versicolor* NRRL 238	86.7	84.9	85.6	81.8	88.2	86.2	79.0	78.8	93.6	91.9	97.0	100	
13	*A. nidulans* ATCC 10074	87.2	82.1	85.0	82.7	86.9	85.0	79.5	79.4	95.3	94.8	95.9	95.3	100

[a] Values were calculated from pairwise alignments using sequence data determined in this study (for GenBank accession numbers, see Table 1) and the STRETCHER algorithm. Species numbers were assigned arbitrarily.
[b] Sequences were derived from type strains or from non-type strains (*A. flavus* ATCC 11497, *A. niger* ATCC 1015, and *A. restrictus* NRRL 148) that exhibited perfect sequence identity with corresponding type strain reference data (15).

occurred over 209 aligned positions) (Fig. 1). Closely related taxa differed by ≤3 nt in the D2 region, e.g., *A. granulosus* versus *A. ustus* (1 nt) and *A. sydowii* versus *A. versicolor* (3 nt). Intraspecies sequence variability was ≤2 nt over the entire D1-D2 region (*A. flavipes*, *A. sydowii*, and *A. nidulans* differed by 1 to 2 nt from their respective type strains; remaining species differed by 0 nt).

In comparison, Fig. 2 shows DNA sequence alignments for the ITS1 and ITS2 regions of the *Aspergillus* strains investigated. In contrast to the D1-D2 regions, the ITS1 sequence alignment revealed five regions with significant interspecies divergence (ITS1 variable regions 1 to 5) (Fig. 2). The diversity within these regions was the result of nucleotide polymorphisms as well as differences in sequence length between some groups of species studied. The most prominent difference in sequence length was noted in ITS1 variable region 3, which exhibited a significant deletion (36 to 49 aligned positions) in the strains representing *A. chevalieri* and the subgeneric group *Nidulantes* (*A. nidulans*, *A. versicolor*, *A. sydowii*, *A. ustus*, and *A. granulosus*). In addition, signature nucleotide sequence patterns could be discerned among closely related species within each of the five variable regions and throughout the remaining parts of the ITS1 alignment; e.g., among *A. granulosus* and *A. ustus*, and among *A. sydowii* and *A. versicolor* (Fig. 2). Intraspecies sequence variability, if present, was ≤3 nt (*A. flavipes*: 3 nt, <2%; *A. niger*: 3 nt, <2%; *A. nidulans*: 2 nt, <2%) except for *A. ustus* strains that differed from the type strain sequence by 11 to 14 aligned positions in the ITS1 region.

Alignment of ITS2 sequences revealed significant interspecies divergence in the area proximal to the 5′ end (ITS2 variable region 1) and at the 3′ end (ITS2 variable region 2) (Fig. 2). These regions varied more in nucleotide polymorphisms than in sequence length, in contrast to the ITS1 variable regions; however, both ITS2 variable regions demonstrated significant sequence length differences between unrelated species compared to related species. As noted for the ITS1 region, signature nucleotide sequence patterns could be identified among closely related species throughout the ITS2 region, especially within its variable region 2 (Fig. 2). Intraspecies nucleotide variability, if present, was ≤5 nt (*A. ustus*, 5 nt, <3%; *A. nidulans* and *A. niger*, 1 nt, <1%; remaining species, 0 nt), except for *A. flavipes* strains that differed from the type strain sequence by 4 to 8 aligned nucleotides in the ITS2 region.

Comparative GenBank analysis of D1-D2, ITS1, and ITS2 ribosomal sequences. To determine the usefulness of a public database to serve as a guide to species identification by comparative DNA sequence analysis, D1-D2, ITS1, and ITS2 sequences for each strain of the 13 *Aspergillus* species studied were used to conduct BLAST searches of the GenBank database (Table 5). All BLAST search results, using our own D1-D2 data as the query sequences, gave top-ranking scores (100% sequence identity) with corresponding reference sequences (35). GenBank searches revealed that sequences for strains of *A. flavipes*, *A. sydowii*, and *A. versicolor* were clearly distinct from those of other *Aspergillus* species or related taxa (i.e., ≥4 nt or ≤99.3% similarity with any other GenBank entry for a different species); however, sequences for the remaining species (*A. candidus*, *A. chevalieri*, *A. flavus*, *A. fumigatus*, *A. granulosus*, *A. nidulans*, *A. niger*, *A. restrictus*, *A. terreus*, and *A. ustus*) were found to be identical with, or very similar to, reference sequences from closely related but different taxa (molecular siblings) (Table 5). For instance, *A. flavus* D1-D2 sequences were not distinguished from sequences representing *Aspergillus oryzae*, *Aspergillus parasiticus*, *Aspergillus sojae*, *Aspergillus subolivaceus*, or *Aspergillus terricola* (100% identity in the D1-D2 region, Table 5). Only 1 nt difference (99.8% identity) was observed between our D1-D2 sequences for strains of *A. flavus* and GenBank entries for *Aspergillus tamari* and *Aspergillus flavofurcatus* (Table 5). In total, 8 of the 13 *Aspergillus* species examined demonstrated identical D1-D2 sequences with at least one molecular sibling and the remainder of the species gave sequence identities of 99.3 to 99.8% with molecular siblings (Table 5).

All BLAST results, using our own ITS1 and ITS2 data as the query sequences, gave top-ranking scores (100% sequence identity) with corresponding GenBank reference sequences; the only exception was for *A. granulosus* because no sequences for the ITS regions were available for this species at the time of our study. Although our sequences usually showed 100% identity with GenBank reference data for *Aspergillus* type strains, if available (15), some variations were detected among sequevars; however, these differences did not change the species identification of a given sequevar based on GenBank search data nor did it alter the overall composition of top-ranking matches, i.e., the list of molecular siblings. Therefore, BLAST results for sequevars other than those representing type strains were not included in Table 5.

Unlike GenBank search results using query sequences from the D1-D2 region, where 8 of the 13 species gave 100% identical sequence data for at least one molecular sibling and all remaining species gave sequence similarities of 99.3% to 99.8% with their respective molecular siblings (Table 5), ITS1 sequences gave 100% identity with molecular siblings for only 4 of the 13 *Aspergillus* species studied (*A. chevalieri*, *A. flavus*, *A. nidulans*, and *A. niger*); the remaining species gave sequence similarities ranging from 84.3% (*A. granulosus*) to 99.4% (*A. restrictus*) with sibling species (Table 5). ITS2 sequences gave 100% identity with molecular siblings for only 5 of the 13 *Aspergillus* species studied (*A. chevalieri*, *A. flavus*, *A. nidulans*, *A. niger* and *A. ustus*); the remaining species gave sequence similarities ranging from 91.5% (*A. terreus*) to 99.4% (*A. niger*) with sibling species (Table 5). However, it must be noted that ITS reference data were not available in GenBank for all sibling species, especially for *Eurotium*, *Emericella*, and *Neosartorya* species, at the time of our study.

Assessment of the quality of D1-D2, ITS1 and ITS2 sequence entries in GenBank. Inspection of BLAST alignments, and of multiple alignments generated with *Aspergillus* species D1-D2, ITS1, and ITS2 data from GenBank, revealed that many *Aspergillus* species sequences in this database had truncated ends and/or heterogeneities at positions found to be conserved at the generic or subgeneric level among the reference sequences of type strains and of authenticated culture collection strains (15, 35). These data indicate that many of the current GenBank sequences are not complete and/or contain errors in sequence data for the ribosomal regions studied. Further, BLAST search analysis of some query sequences gave best-match results with reference sequences of corresponding type strains but also resulted in excellent scores for sequences

```
                             5'end of D1
                             ↓
A. fumigatus ATCC 1022       CCCCTTCGGGGTCCGCGTTGTAATTTGCAGAGGATGCTTCGGGTGCAGCCCCCGTCTAAGTGCCCTGGAACGGGCCGTCATAGAGGGTGAGAATC
A. flavus ATCC 11497         .T..............A.............................G.....T...........................G..............
A. niger ATCC 1015           .T.......A......A......................T......G.................................G..............
A. niger ATCC 16404          .T.......A......A......................T......G.................................G..............
A. terreus ATCC 1012         .T..............A..............................................................................
A. flavipes ATCC 11013       .....C..........A.....................................................T.........G..............
A. flavipes ATCC 16805       .....C..........A.....................................................T.........G..............
A. flavipes ATCC 24487       .....C..........A.....................................................T.........G..............
A. candidus NRRL 303         .T..............A.............................G.....T......................T....G..............
A. restrictus NRRL 148       .T.............A...........T..................G................T......T.....A...G..............
A. chevalieri ATCC 16443     .....C.........A...........T..................G.............................A..GG..............
A. granulosus NRRL 1932      .....C.........A..............................G.....T...........................G..............
A. ustus ATCC 14417          .....C.........A..............................G.....T...........................G..............
A. ustus ATCC 16801          .....C.........A..............................G.....T...........................G..............
A. ustus NRRL 275            .....C.........A..............................G.....T...........................G..............
A. sydowii NRRL 250          .....C.........A..............................G.....T...........................G..............
A. sydowii NRRL 254          .....C.........A..............................G.....T...........................G..............
A. versicolor NRRL 238       .....C.........A..............................G.....T...........................G..............
A. nidulans ATCC 10074       ..............................................G.....T...........................G..............
A. nidulans ATCC 16855       ..............................................G.....T...........................G..............
A. nidulans CDC B6597        ..............................................G.....T...........................G..............

                                                                3'end of D1                       5'end of D2
                                                                          ↓                                 ↓
A. fumigatus ATCC 1022       CCGTCTGGGACGGGGTGTCTGCGTCCGTGTGAAGCTCCTTCG< 28S rRNA gene conserved region >CGTTTGCGACCAGACTCGC
A. flavus ATCC 11497         ..........T........C...C..................                                  ..C................
A. niger ATCC 1015           ......T..G.........C.T.C......A...........                                  ..C................
A. niger ATCC 16404          ......T..G.........C.T.C......A...........                                  ..C................
A. terreus ATCC 1012         ....A....G................................                                  ..C....A...........
A. flavipes ATCC 11013       ..........T......C...T.C..................                                  ..C................
A. flavipes ATCC 16805       ..........T......C...T.C..................                                  ..C................
A. flavipes ATCC 24487       ..........T......C.....C..................                                  ..C................
A. candidus NRRL 303         ..........T........C...C..................                                  ..C................
A. restrictus NRRL 148       .........G.........C......................                                  .AC..A.............
A. chevalieri ATCC 16443     ...................C......A...............                                  ..C................
A. granulosus NRRL 1932      ......T..G.A.....C.C.T.C..................                                  ..C...............G
A. ustus ATCC 14417          ......T..G.A.....C.C.T.C..................                                  ..C...............G
A. ustus ATCC 16801          ......T..G.A.....C.C.T.C..................                                  ..C...............G
A. ustus NRRL 275            ......T..G.A.....C.C.T.C..................                                  ..C...............G
A. sydowii NRRL 250          ......T..G.A.....C.C.T.C..................                                  ..C....A..........G
A. sydowii NRRL 254          ......T..G.A.A...C.C.T.C..................                                  ..C....A..........G
A. versicolor NRRL 238       ......T..G.A.....C.C.T.C..................                                  ..C...............G
A. nidulans ATCC 10074       ......T..G.A.....C.C.T.C..................                                  ..C...............G
A. nidulans ATCC 16855       ......T..G.A.....C.C.T.C..................                                  ..C...............G
A. nidulans CDC B6597        ......T..G.A.....C.C.T.C..................                                  ..C...............G

A. fumigatus ATCC 1022       CCGCGGGGTTCAGCCGGCATTCGTGCCGGTGTACTTCCCCGTGGGCGGGCCAGCGTCGGTTTGGGCGGCCGGTCAAAGGCCCTCGGAATGTATCA
A. flavus ATCC 11497         .TC.A..................................T.G......................................T.C.........GTG
A. niger ATCC 1015           ..................................................................................CT........GTG
A. niger ATCC 16404          ..................................................................................CT........GTG
A. terreus ATCC 1012         T................GC....GC................C.......................................TC.........G.G
A. flavipes ATCC 11013       .TC......................................G.......................................TC.........G.G
A. flavipes ATCC 16805       .TC......................................G.......................................TC.........G.G
A. flavipes ATCC 24487       ..C......................................G.......................................TC.........G.G
A. candidus NRRL 303         ............................................................................GC..T.C.........G.G
A. restrictus NRRL 148       T.A...............T....G.................G.A.............................T........CT........A..
A. chevalieri ATCC 16443     TTC...............T....G.................G........................................CT........A.G
A. granulosus NRRL 1932      ..C............A..........T..............G........................................CA.......G..G
A. ustus ATCC 14417          ..C............A...C......T..............G........................................CA.......G..G
A. ustus ATCC 16801          ..C............A...C......T..............G........................................CA.......G..G
A. ustus NRRL 275            ..C............A...C......T..............G........................................CA.......G..G
A. sydowii NRRL 250          ..T............A..........T..............G..C.....................................CA..........G
A. sydowii NRRL 254          ..T............A..........T..............G..C.....................................CA..........G
A. versicolor NRRL 238       ..T............A..........T..............G..C................C....................CA..........G
A. nidulans ATCC 10074       ..C............A...C......T..............G........................................CA..........G
A. nidulans ATCC 16855       ..C............A...C......T..............G........................................CA..........G
A. nidulans CDC B6597        ..C............A..........T..............G........................................CA..........G

                                                                                                       3'end of D2
                                                                                                                 ↓
A. fumigatus ATCC 1022       CCTCTCGGGGT-GTCTTATAGCCGAGGGTGCAATGCGGCCTGCCTGGACCGAGGAACGCGCTTCGGCTCGGACGCTGGCGTAATGGTCGTAAATG
A. flavus ATCC 11497         ..CTC.....C-AC..........G.A.............A..........................A...........A.............C.
A. niger ATCC 1015           ..CTC.....C-AC.........AG...............A..........................A...........A.............C.
A. niger ATCC 16404          ..CTC.....C-AC.........AG...............A..........................A...........A.............C.
A. terreus ATCC 1012         ..CT......C-.C..........G...............A..........................A...........A.......T.....C.
A. flavipes ATCC 11013       ..CT......C-.C..........G...............A......................................A.............C.
A. flavipes ATCC 16805       ..CT......C-.C..........G...............A......................................A.............C.
A. flavipes ATCC 24487       ..CT......C-.C..........G...............A......................................A.............C.
A. candidus NRRL 303         ..........C-.C..........G.......G.......A..........................A...........A.............C.
A. restrictus NRRL 148       ..C........-...........AG......C........A..CA......................A...........A...............
A. chevalieri ATCC 16443     ..........C-.C.........AG.....TC........A..........................A...........A.............C.
A. granulosus NRRL 1932      ..CTC.....C-...........TG...............A..C.......................A.....................C...C.
A. ustus ATCC 14417          ..CTC.....C-...........TG...............A..C.......................A.....................C...C.
A. ustus ATCC 16801          ..CTC.....C-...........TG...............A..C.......................A.....................C...C.
A. ustus NRRL 275            ..CTC.....C-...........TG...............A..C.......................A.....................C...C.
A. sydowii NRRL 250          T.CTC....AC-...........TG...............A..........................A.....................C...C.
A. sydowii NRRL 254          T.CTC....AC-...........TG...............A..........................A.....................C...C.
A. versicolor NRRL 238       T.CTC....AC-...........TG...............A..........................A...................T.C...C.
A. nidulans ATCC 10074       ..CTC......T...........TG...............A..C.......................A.....................C...C.
A. nidulans ATCC 16855       ..CTC......T...........TG...............A..C.......................A.....................C...C.
A. nidulans CDC B6597        ..CTC......T...........TG...............A..C.......................A.....................C...C.
```

assigned to (i) unrelated or distantly related taxa, i.e., *Cordyceps sinensis* AJ488259, AJ488260, and AJ488268 (*A. chevalieri* query), *Aureobasidium mansonii* AF121288 (*A. flavus* query), and *A. wentii* U03522 and U03523, *Arthrobotrys* species U72602, *Gliocladium cibotii* AF021264, AF048739, and AF048738, and *Verticillium bulbillosum* AF048741 (*A. niger* query), or (ii) more closely related but different taxa, i.e., *A. nidulans* AF455499 (*A. sydowii* query), *A. nidulans* AF455424, AF455505, and AY452983 (*A. versicolor* query), and *A. sydowii* AJ312221 (*A. nidulans* query). When reference alignments were used for type strains, all of these GenBank entries were shown to exhibit significant nucleotide differences compared to respective reference sequences. These results indicate the existence of mislabeled ribosomal *Aspergillus* species data in GenBank ("false" molecular siblings). We could not determine, however, whether these ambiguous sequence annotations were due to misnamed or misidentified isolates or to manipulation or editing errors occurring during sequence analysis or GenBank submission.

DISCUSSION

The increasing popularity of molecular approaches for the identification of fungal pathogens reflects significant improvements in DNA analysis in recent years, including the development of broad-range (panfungal) primers, kit-based automated sample processing and DNA amplification systems, and expanding public and private DNA sequence databases. The most promising results were derived from ribosomal sequencing studies examining yeasts and dermatophytes; however, dimorphic fungi and moulds are also being investigated in order to overcome diagnostic problems encountered by extended incubation periods and the plasticity of traditional identification criteria (5, 13, 14, 18, 27, 28, 40). An important prerequisite for the development of clinical molecular approaches is, of course, the estimation of the target resolution at the species level using a comprehensive collection of reference strain sequences. Unfortunately, such an assessment has not been published to date for the various ribosomal targets of *Aspergillus* species. Therefore, we evaluated the utility of the ribosomal D1-D2, ITS1, and ITS2 sequences as targets for the differentiation of medically important *Aspergillus* species by comprehensive DNA sequence analysis of type cultures and authenticated culture collection reference strains and conducted a systematic comparison of these sequences to those available in the GenBank database.

Primary structural analysis of our *Aspergillus* species D1-D2 sequences revealed negligible intraspecies variability and recognizable interspecies divergence within the D2 region as described for other fungi (12, 24, 25, 36, 42). Although comparison of the D1-D2 sequences determined in this study with those in GenBank showed complete identity with corresponding reference data (35), most BLAST search results were inconclusive at the species level due to identical or very similar (>99% identical) reference data which were assigned to other organisms than the query sequences (molecular siblings). Nonetheless, these sibling organisms most frequently belonged to the same *Aspergillus* group as the query species (35) and supported the identification to at least the subgeneric group level. Consequently, results obtained by sequencing the D1-D2 region should be interpreted accordingly. The same test specificity applies to currently available large-subunit rRNA gene probes (42) which target D2 sequences conserved among medically important species and their molecular siblings. In addition, other targets with conserved interspecies sequences are expected to exhibit analogous diagnostic limitations, such as those reported previously using the mitochondrial cytochrome *b* gene (46, 58).

Unlike the D1-D2 region, the two ITS regions investigated were found to exhibit localized hypervariable regions containing most of the intraspecies sequence diversity. Nonetheless, most ITS amplicons showed a consistent sequence length among strains of the same species in one or both spacer regions; exceptions included sequevars of *A. flavipes*, *A. nidulans*, *A. niger*, and *A. ustus*. These hypervariable regions also contained most of the interspecies sequence diversity; however, similar sequence lengths in one or both spacer regions often occurred, making the overall size of ITS amplicons an imperfect tool to differentiate medically important *Aspergillus* species. This was especially evident when data from only one spacer region were analyzed. It is also not known if sibling species or other potential pathogens possess similar amplicon lengths that would confound a specific identification because, at present, GenBank data are incomplete in the ITS region. Our work therefore supports a previous report suggesting that *A. fumigatus* and *A. flavus* could not be differentiated by ITS amplicon length alone and that application of a specific DNA capture probe may be required for species identification (16). Alternatively, ITS amplicons have been characterized using single-strand conformation polymorphism (SSCP) analysis exploiting both size and sequence differences (23, 56); however, clinical diagnostic applications may be compromised by intraspecies SSCP pattern variability such as that described recently for strains of *A. fumigatus* and *A. flavus* (39). Multiple SSCP patterns are also predicted to occur within species of *A. flavipes*, *A. nidulans*, *A. niger*, *A. ustus*, and other *Aspergillus* species that exhibit sequence differences among strains of the same species (this study; 15, 34, 44, 53, 54).

The presence of intraspecies ITS variability did not hamper *Aspergillus* species identification by comparative sequence analysis, as indicated by similar BLAST search results for all sequevars of a particular species. All ITS sequences determined in this study yielded top-ranking BLAST scores with corresponding reference data (15, 34) regardless of the spacer region analyzed (ITS1 or ITS2) except for *A. granulosus* because its ITS sequence was not available in GenBank at the

FIG. 1. Alignment of complete *Aspergillus* D1-D2 regions illustrating the sequence divergence among medically important species. The highly conserved region of the 28S rRNA gene intervening between the D1 and D2 regions has been omitted. Dots indicate identical nucleotides compared to the leader sequence (*A. fumigatus* strain ATCC 1022); dashes indicate alignment gaps. Sequence data were derived from this study (for GenBank accession numbers, see Table 1).

```
                                5'end of ITS1
                                ↓
A. fumigatus ATCC 1022          CCGAGTG-AGGGC--CCTCTGG----GTCCAACCTCCCACCCGTGTCTATC-GTACC-TTGTTGCTTCGGCGGGCCCGCC-GTTTC-GACGGCCGCCGGGGAG--
A. flavus ATCC 11497            .......T....-TT...AGC.A---.C..................T..-.T.....T.A.....................--A..--.T...........G---
A. niger ATCC 1015              .......C-...--T...T..----.C...........T..........-T.....-C.....................-.C..--.T............G.GC
A. niger ATCC 16404             .......C-...--T...T..----.C...........T..........-T.....-C.....................-.C..--.T............G.GC
A. terreus ATCC 1012            .......C-...--T.T.TAT.----.C.................A....-T.....-......................A.CG.T-.CT..........G.--
A. flavipes ATCC 11013          .......-....--T....GT.----.C.................A...-.T....AC.....................A.CG..C.CT..........G.--
A. flavipes ATCC 16805          .......-....--T....GT.----.C.................A...-.T....AC.....................A.CC.A-.CT..........G.--
A. flavipes ATCC 24487          .......-....--T....GT.----.C.................A...-.T....AC.....................A.CGC.C.CT..........G.--
A. candidus NRRL 303            .......-....-TTT.....AA---.C..................A..C.-.....-C.....................----..--.............GA--
A. restrictus NRRL 148          .......C-....--.......----.............T.........-CTT....-C.....................---...--.T...........G.--
A. chevalieri ATCC 16443        .......C-....--.......----.............T............T....-C...............------------------------------
A. granulosus NRRL 1932         .......C...T.TG..C....GCAG.C.T..............AA..C.T.-...AAC.........------------------------------
A. ustus ATCC 14417             .......C...T.TG..C.C..GCAG.C.T..............AA..C.T.-...AAC.........------------------------------
A. ustus ATCC 16801             .......C...T.TG..C.C..GCAG.C.T..............AA..C.T.-...AAC.........------------------------------
A. ustus NRRL 275               .......C...T.TG..C.C..GCAG.C.T..............AA..C.T.-...AAC.........------------------------------
A. sydowii NRRL 250             .T.....C-....TG....C..GC--.C...............AA..C.T--.A.AC..........------------------------------
A. sydowii NRRL 254             .T.....C-....TG....C..GC--.C...............AA..C.T--.A.AC..........------------------------------
A. versicolor NRRL 238          .T.....C-....TG....C..GC--.C...............A...C.T--.A.AC..........------------------------------
A. nidulans ATCC 10074          .......C-....TG....C..GC--.C...............A...C.T--.A.AC..........------------------------------
A. nidulans ATCC 16855          .......C-....TG....C..GC--.C...............A...C.T--.A.AC..........------------------------------
A. nidulans CDC B6597           .......C-....TG....C..GC--.C...............A...C.T--.A.AC..........------------------------------
                                       ITS1 variable region 1          ITS1 variable region 2        ITS1 variable region 3 (5'half)

                                                                                                                        3'end of ITS1
                                                                                                                                    ↓
A. fumigatus ATCC 1022          GCCTTGCGCCCCC---GGGCCCGCGCCCGCCGAAGACCCCAACAT--GAACGCTGT-TCTGA-AAGT-ATGCAGTCTGAGT-TGATTAT---CGTAATC-AGTTA
A. flavus ATCC 11497            -.TC.CA....----...............G...---.C..C--....T....--....-TCTAG-..A.........-.....G.AT-..C....-.....
A. niger ATCC 1015              ....-CT.....C--......T.......G..........C--....A....--....-...CG-...........-.....GAAT--.C....-.....
A. niger ATCC 16404             ....-CT.....C--......T.......G..........C--....C....--....-...CG-..........-C....G.TT--.C....-.....
A. terreus ATCC 1012            -.GACT......---......T.......G...........--....C....-.....-...CT-...........G.....C.TT--.C....-.....
A. flavipes ATCC 11013          --...CT.....---......T.......G..........C--....A....T.....-...CC-..T.---...A.TC....C.TT--.......-.....
A. flavipes ATCC 16805          --...CT.....---..............G..........C--....A....T.....-...CC-..T.---...A.CC....C.TT--.......-.....
A. flavipes ATCC 24487          --...CT.....---......T.......G..........C--....A....T.....-...CC-..T.---...A.CC....C.TT--.......-.....
A. candidus NRRL 303            -...C...T...---.........................C--....A....--....A...---..............-C....G.TA--CC.....-....C.
A. restrictus NRRL 148          --...CT.....---..............G....T.......T-....A....--.....--..GT-..........T.TCA...AAGAAA....--.....
A. chevalieri ATCC 16443        ----------------T....ACG.......G....T--.....TT........--.......--...T-...........T.TTA-G.TA-AAC....--.....
A. granulosus NRRL 1932         ----........TCC-...GG.-A.-.....G.....A..C.----....CTCT.G.---TT...CC-..TT.......CT....A-----GCA....T.-...
A. ustus ATCC 14417             ----.........CC-...GGT-A.-.....G.....A..C.----....CTCC.G..--TTT.---..TT.......CT....A-----GCA..C.T.-...
A. ustus ATCC 16801             ----........TCC-...GGT-A.-.....G.....A..TT----....CTCT.G..--TTT.---..TT.......CT....A-----GCA..C.T.-...
A. ustus NRRL 275               ----.....T...CCC...GG.-A.-.....G.....A..C.----....CTCT.G.---TAT..CG-..TC.......CT....A--CAAGCA..C.T.A...
A. sydowii NRRL 250             ----G.AA.....TC-...GG..A.-.....GG...TA.T------....TTCA.GC....-G...G............C...A...----AA....-...C.
A. sydowii NRRL 254             ----G.A..T...TC-...GG..A.-.....GG...TA.T------....TTCA.GC....-G...G............C...A...----AA....-...C.
A. versicolor NRRL 238          ----G.A....T.TC-...GG..A.-.....GG...TA.T------....TTCA.GC....-G...G............C...A...----AA....-...C.
A. nidulans ATCC 10074          ----G.A......CA-...G-..A.-.....GG....A.T------....TTCA.GC....-G...G...........CC...A..C-----A....-...C.
A. nidulans ATCC 16855          ----G.A......TA-...G-..A.-.....GG....A.T------....TTCA.GC....-G...G...........CC...A..C-----A....-...C.
A. nidulans CDC B6597           ----G.A......CA-...GG..A.-.....GG....A.T------....TTCA.GC....-G...G...........CC...A..C-----A....-...C.
                                ITS1 variable region 3 (3')            ITS1 variable region 4                 ITS1 variable region 5

                                                         5'end of ITS2
                                                         ↓
A. fumigatus ATCC 1022          <   5.8S rRNA gene      >GCTGCCC-TCAAGCACGGCTTGTGTGTTGGGCCCCCGTCCCC---CTCT-CCCGGGGGACGGGCCCGAAAGGCAGCGGCGGCA
A. flavus ATCC 11497                                     .......A.....................T.GT......----...C.GG............C................
A. niger ATCC 1015                                       .......-......C..............T.G.......---....-..G...............................
A. niger ATCC 16404                                      .......-......C..............T.G.......--T....-..G...............................
A. terreus ATCC 1012                                     .......-......C.................T.......--CGG..-..................................
A. flavipes ATCC 11013                                   A......-......C.......A--.....T..T.......-----..C................................
A. flavipes ATCC 16805                                   A......-......C.......A--.....T..T.......----------.C............................
A. flavipes ATCC 24487                                   A......-......C.......A--.....T..T.......--------................................
A. candidus NRRL 303                                     .......-........................-.......---GGTAC...C..............................
A. restrictus NRRL 148                                   .......-...............................GGTT..T.AAG.C-............................
A. chevalieri ATCC 16443                                 .......-......................TT.......------TGG.AAC............A........T.......
A. granulosus NRRL 1932                                  .......T......C..............T.GT......--------..................................
A. ustus ATCC 14417                                      .......T......C..............T.GT......--------..................................
A. ustus ATCC 16801                                      .......T......C..............T.GT......--------T.................................
A. ustus NRRL 275                                        .......T......C..............T.GT......--------..................................
A. sydowii NRRL 250                                      .......A......C..............T.GT......--------..................................
A. sydowii NRRL 254                                      .......A......C..............T.GT......--------..................................
A. versicolor NRRL 238                                   .......A......C..............T.GT......--------..................................
A. nidulans ATCC 10074                                   .......-......C..............T.GT......--------..................................
A. nidulans ATCC 16855                                   .......-......C..............T.GT......--------..................................
A. nidulans CDC B6597                                    .......-......C..............T.GT......-------C..................................
                                                                                          ITS2 variable region 1

                                                                                                                        3'end of ITS2
                                                                                                                                    ↓
A. fumigatus ATCC 1022          CCGCGTCC-GGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTC--TGTAGGCCCGGCCGG-CGCCAGCCGAC--------ACCCA-AC--TTTATTTTCTAAGG
A. flavus ATCC 11497            ........-.A........................C...--................-...TT....---------..G..A.TC--AATC....TCC...
A. niger ATCC 1015              ........-.A.......................A....--......ATT......-....T.....G-----TTTT...-..C----...C..TCC...
A. niger ATCC 16404             ........-.A.......................A....--......ATT......-....T.....G-----TTTT...-..C----...C..TCC...
A. terreus ATCC 1012            ........-...................C...TT.C....--C..............-....C.....GCATTTATTTG..-..--..GT....TCC...
A. flavipes ATCC 11013          ........-..........................C...--................-........C..G----------..-..--ACCT.....T.C...
A. flavipes ATCC 16805          ........-..........................C...--................-........C..G----------..G.TC-A.CC.....T.C...
A. flavipes ATCC 24487          ........-..........................C...--................-........C..G----------..C.TC-A.CC.....T.C...
A. candidus NRRL 303            ........-..........................C...--..C.............-...........C------A.....-..C----......TAC...
A. restrictus NRRL 148          ...T....-..........T.........C.....CA...G-......T.......CG.......----------TCTT..-..CAAAC-....AACC...
A. chevalieri ATCC 16443        ..AT...-T..........................C...C-C.....T..A..T..CA..T....------------T.G..-..CAA.CT....AACC...
A. granulosus NRRL 1932         ........T..........................C...GA.-....G.......G...........G------TCT...-..C-...TA....ACC...
A. ustus ATCC 14417             ........-..........................C...GA.-....G.......G.........G.G------TCT...-..C-..CTA....ACC...
A. ustus ATCC 16801             ........-..........................C...GA.-....G.......G.........G.G------TCT...-..C-...TA....ACC...
A. ustus NRRL 275               ........T..........................C...GT.-....G.......G.........G.G------TCT...A..C-...TA....ACC...
A. sydowii NRRL 250             ...T....-..........................C...GAC-....G.......G...........G------TCT...-..C---AT....CT.C...
A. sydowii NRRL 254             ...T....-....................A.....C...GAC-....G.......G...........G------TCT...-..C---AT....CT.C...
A. versicolor NRRL 238          ...T....-..........................C...GA.T....G.......G...........G--------T...-..C---AT....CT.C...
A. nidulans ATCC 10074          ...T....-..........................C...GA.-....G.......G.........G.G------TCT...-..C--..........C...
A. nidulans ATCC 16855          ...T....-..........................C...GA.-....G.......G.........G.G------TCT...-..C--..........C...
A. nidulans CDC B6597           ...T....-..........................C...GA.-....G.......G.........G.G------TCT...-..C--..........C...
                                                                                        ITS2 variable region 2
```

TABLE 5. Molecular siblings of medically important *Aspergillus* species investigated in this study

Query species[a]	Molecular sibling[b]	Maximum sequence identity (%) per target (minimum nucleotide position difference)[c]		
		D1-D2	ITS1	ITS2
A. candidus	*A. campestris*	100 (0)	NA[d]	NA
A. (*Eurotium*) *chevalieri*	Various *Eurotium* species[e]	100 (0)	100 (0)	100 (0)
A. (*Fennellia*) *flavipes*	None			
A. flavus	*A. oryzae*	100 (0)	100 (0)	100 (0)
	A. parasiticus	100 (0)	97.8 (4)	97.7 (4)
	A. sojae	100 (0)	97.8 (4)	97.7 (4)
	A. tamarii	99.8 (1)	95.6 (8)	97.0 (5)
	A. subolivaceus	100 (0)	NA	NA
	A. terricola	100 (0)	NA	NA
	A. flavofurcatus	99.8 (1)	NA	NA
A. fumigatus	Various *Neosartorya* species[e]	99.8 (1)	98.9 (2)	97.0 (5)
A. granulosus	*A. ustus*	99.8 (1)	94.3 (9)	98.3 (3)
	A. puniceus	99.6 (2)	84.3 (27)	98.3 (3)
	A. pseudodeflectus	99.8 (1)	NA	NA
A. (*Emericella*) *nidulans*	Various *Emericella* species[e]	100 (0)	100 (0)	100 (0)
A. niger	*A. awamori*	NA	100 (0)	100 (0)
	A. foetidus	NA	100 (0)	100 (0)
	A. phoenicis	100 (0)	98.9 (2)	99.4 (1)
A. restrictus	*A. caesiellus*	99.8 (1)	99.4 (1)	96.5 (6)
	A. conicus	100 (0)	NA	NA
A. sydowii	*A. versicolor*	99.1 (5)	98.1 (3)	98.2 (3)
A. terreus	*Fennellia nivea*	100 (0)	92.6 (14)	91.5 (15)
	A. carneus	100 (0)	NA	NA
	A. allahabadii	99.8 (1)	NA	NA
A. ustus	*A. granulosus*	99.6 (2)	92.5 (12)	98.3 (3)
	A. puniceus	100 (0)	89.5 (18)	100 (0)
	A. pseudodeflectus	99.8 (1)	NA	NA
A. versicolor	*A. sydowii*	99.3 (4)	98.1 (3)	98.8 (2)

[a] See Table 1 for the origin of strains used and for the corresponding GenBank sequence accession numbers; sequence data for only the type strains or reference strains with identical ribosomal sequences compared to corresponding type strains are shown for ease of presentation because despite some nucleotide variations among sequevars of a given species, molecular siblings captured from GenBank were the same for all sequevars within a given species (see Results section).

[b] Organism(s) assigned to a different species than query sequence although exhibiting identical or very similar (>99% identity) sequences in at least one ribosomal region investigated in this study. The listing is limited to *Aspergillus* species currently sanctioned by the International Commission of *Penicillium* and *Aspergillus* (37). Note that the query sequences of *A. chevalieri*, *A. flavus*, *A. nidulans*, *A. niger*, *A. sydowii*, and *A. versicolor* also revealed "false" molecular siblings in GenBank, i.e., GenBank sequences with ambiguous classification as detailed in the Results section of this study.

[c] Comparison between query sequences (this study) and reference sequences of molecular siblings obtained from GenBank (15, 35) and the present investigation (*A. granulosus* ITS sequences) using BLAST and GCG algorithms.

[d] NA, no corresponding reference data available for this molecular sibling in this target region at the time this study was conducted.

[e] Query sequences matched multiple species of this genera in the D1-D2 and ITS regions although ITS reference data for many species of this taxonomic group were not available in GenBank at the time this study was conducted.

time of this study. Reference sequences of organisms most closely related to, but different from, the query species usually exhibited less than 99% sequence identity in at least one spacer region. Nonetheless, some ITS analyses were inconclusive at the species level because similar GenBank reference sequences existed for different organisms (molecular siblings of *A. chevalieri*, *A. flavus*, *A. nidulans*, and *A. niger*). Additional ambiguity arose from the existence of probably misclassified or misnamed GenBank sequences exhibiting excellent BLAST scores with some of our query sequences (*A. chevalieri*, *A. flavus*, *A. nidu-*

FIG. 2. Alignment of complete *Aspergillus* ITS1-ITS2 regions illustrating the sequence divergence among medically important species. The highly conserved intervening 5.8S rRNA gene has been omitted. Dots symbolize identical nucleotides compared to the leader sequence (*A. fumigatus* strain ATCC 1022); dashes indicate alignment gaps. Sequence data were derived from this study (for GenBank accession numbers, see Table 1).

lans, *A. niger*, *A. sydowii*, and *A. versicolor*) and yet assigned to different taxa (including *Arthrobotrys*, *Aspergillus*, *Aureobasidium*, *Cordyceps*, *Gliocladium*, and *Verticillium* species—"false" molecular siblings [see Results]). Truncated and low-quality GenBank sequences were disregarded in the present study and therefore did not affect identification; however, truncated and low-quality GenBank entries clearly limit the utility of this database to assess inter- and intraspecies sequence similarity. Overall, ITS comparative sequence analysis, which included both spacer regions, showed better species differentiation than use of a single spacer region alone. However, differentiation between some more closely related *Aspergillus* species, especially among *Eurotium* and *Emericella* species, may require analysis of less conserved targets such as the ribosomal external transcribed spacer regions or intergenic spacer regions (18).

Considering the results presented above, the development of ribosomal approaches targeting *Aspergillus* groups rather than particular species would seem more practical. Ribosomal sequence analysis of the 13 medically important species investigated in this study allowed unambiguous identification of 11 distinct groups (*A. flavipes* and remaining groups as listed in the Results section) using either the D1-D2 or the ITS regions. Further, these molecular groups largely reflected relationships established previously by phenotypic classification (38, 45). Indeed, *A. fumigatus* group-specific primers (64), various sets of species-specific primers and group-specific probes (15), or panfungal primers in combination with group-specific probes (6, 42) may provide sufficient differentiation for diagnostic applications in a conventional clinical setting.

Nevertheless, ribosomal approaches for *Aspergillus* species group identification still requires important improvement in the GenBank database, including submission of additional sequence information for the ITS1 and ITS2 regions for sibling species, implementation of quality control measures for sequence entries using comparisons to known reference sequences, authoritative nomenclatural updates, and expert-based reclassification of mislabeled sequences (correct classification of currently "false" molecular siblings). Accurate sequence databases would not only benefit clinical diagnostic applications but also facilitate the ongoing effort to improve the current taxonomy of the genus *Aspergillus*, which still lacks complete definition by molecular criteria. Indeed, traditional classification and identification of *Aspergillus* species is based on morphological criteria usually expressed in a continuum rather than as discrete entities as discussed in detail by Raper and Fennell (38). To some extent, this may explain the intraspecies ribosomal divergence found in *A. flavipes*, *A. niger*, and *A. ustus* as well as within other species (this study; 15, 33, 34, 35, 44, 53, 54) and the potential diagnostic problems encountered with species-specific probes (30, 32). A true species definition for molecular taxonomic purposes is lacking at present for the aspergilli. Because DNA-DNA hybridization studies are excessively laborious and difficult to perform and interpret, and because many of the medically important *Aspergillus* species studied have no known sexual state (precluding the establishment of a biological species concept based on genetic crosses), other means will need to be employed to define species within the genus *Aspergillus*.

In conclusion, the medically important *Aspergillus* species investigated in this study can largely be identified at the traditional group level using ribosomal D1-D2 or ITS regions as diagnostic targets. Upon major improvement of present sequence databases, identification to the species level should be feasible through ITS sequence analysis; some clinical isolates, however, may require additional analyses. Such improved databases should enable a more accurate identification of *Aspergillus* species, thus promoting further advancements in the molecular diagnosis, epidemiology, and clinical management of invasive aspergillosis.

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