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Identification of *Fusarium* Species Involved in Human Infections by 28S rRNA Gene Sequencing

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***Fusarium* spp. have emerged as major opportunistic fungal agents. Since new antifungal agents exhibit variable activity against *Fusarium* isolates depending on the species, rapid identification at the species level is required. Conventional culture methods are difficult, fastidious, and sometimes inconclusive. In this work, we sequenced a 440-bp fragment encoding the 28S rRNA from 33 *Fusarium* isolates belonging to six *Fusarium* species associated with human infections. The data were then analyzed by the neighbor-joining method. By using distance matrix analysis and constructing the phylogram, we could easily distinguish the different species for all but one isolate. The method also allowed differentiation between the closely related genera *Acremonium* and *Cylindrocarpon*. In contrast to the case with conventional methods, the results could be obtained within 48 h from a 3-day culture and are independent of mycologist experience, making this method rapid and reliable for identification of *Fusarium* species isolated from patients.**

The fungi belonging to the genus *Fusarium* are well-known plant pathogens and food contaminants that also cause superficial and subcutaneous infections, such as onychomycosis and keratomycosis, in humans (9). They have recently emerged as major opportunistic agents in immunocompromised hosts, especially in patients with hemopathy (2, 7). They are now considered the third most common fungal genus (after *Candida* and *Aspergillus*) isolated from systemic infections in bone marrow transplantation patients (8). Four species account for more than 95% of human infections: *Fusarium solani*, *Fusarium moniliforme* (*Fusarium verticilloides*), and *Fusarium oxysporum* are each responsible for about 30% of the cases, whereas *Fusarium dimerum* is involved in 5% of the cases (6, 7). Diagnosis of *Fusarium* at the species level is based on conventional methods, which include the description of colonies on appropriate media (texture, color, and pigment etc.) and microscopic description of conidiogenous cells and conidia. This can be best observed after 2 weeks of incubation, lengthening the time for a definitive diagnosis. Because of important variations of characters, such as pigmentation and growth rate, that are often seen within a given species, only well-trained mycologists are able to ensure the diagnosis (6). The results are thus frequently inconclusive, as seen in various reports where one-third to one-half of the isolates are not identified at the species level (2, 7). Identification at the species level is important for epidemiological purposes and may become absolutely necessary because some new antifungal agents exhibit variable activity against *Fusarium* depending on the species (1, 13). In this work, we report a rapid method for the identification of significant *Fusarium* species involved in human infections by using rRNA gene (rDNA) sequencing.

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MATERIALS AND METHODS

Strains. The fungal isolates used in this work are listed in Table 1. A total of 33 isolates belonging to six different *Fusarium* species were tested, including 18 reference strains and 15 clinical isolates collected from superficial and deep infections. Three clinical isolates and one reference strain belonging to the genus *Acremonium* and two *Cylindrocarpon tonkinense* reference strains were also studied due to the difficulties in distinguishing between these genera and *Fusarium* (6). In addition, nine control isolates from unrelated fungi were included to test the specificity of the designed primers (see below). The identification of *Fusarium* and *Acremonium* isolates was performed according to the colonial aspects and microscopic morphology after 7 and 14 days of culture at 25°C on potato dextrose agar (Difco Laboratories, Detroit, Mich.) plates in daylight. Examination of micro-, meso-, and macroconidia, chlamydospores, and phiallides allowed differentiation between the *Fusarium* species (4, 6). All of the isolates were stored in 10% glycerol at −80°C until tested.

DNA extraction. After a 3-day incubation at 35°C on Sabouraud agar (Sanofi-Diagnostic Pasteur, Marnes la Coquette, France) plates, cultures were discharged in 300 μl of distilled water in a microcentrifuge tube. A volume of 100 μl of Chelex solution (10% [wt/vol] Chelex-100 [Bio-Rad, Hercules, Calif.] in an aqueous solution of 0.1% [wt/vol] sodium dodecyl sulfate sodium salt [Sigma, St. Louis, Mo.], 1% [vol/vol] Nonidet P-40 [Sigma], and 1% [vol/vol] Tween 80 [Sigma]) was added. The tubes were incubated at 95°C for 30 min and then on ice for 5 min. DNA was removed from the supernatant after 5 min of centrifugation (10,000 × *g*) and stored at −80°C until used.

Amplification and sequencing. Two oligonucleotides (Fus1 [5′-TGAAATCT GGCTCTCGGG] and Fus2 [5′-CATGCGCGAACCTCAGTC]) were designed after comparison of *Fusarium* 28S rDNA sequences in the GenBank database. Amplification was performed in a volume of 50 μl with 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 5 μl of DNA, a 0.25 μM concentration of each oligonucleotide primer, 0.2 mM deoxynucleoside triphosphates (Pharmacia, Uppsala, Sweden), 0.3 U of *Taq* Gold (PE Applied Biosystems, Foster City, Calif.), and 5 μl of DNA. Thermal cycling parameters were as follows: 10 min at 94°C, followed by 35 cycles of 30 s at 94°C, 90 s at 64°C, and 90 s at 72°C and a final extension of 10 min at 72°C. The PCR products were separated by electrophoresis and visualized under UV. Amplification products were purified by using microspin S-400 HR purification columns (Pharmacia). The sequence reactions were made with 5 μl of purified amplified DNA, 4 pmol of primers, and 4 μl of Big Dye Terminator (PE Applied Biosystems) according to the manufacturer's instructions. The extension products were then precipitated, washed, and resuspended in Template Suppression Reagent (PE Applied Biosystems). After denaturation at 95°C for 2 min, the samples were loaded onto POP6 capillary columns in a ABI Prism 310 Genetic Analyzer (PE Applied Biosystems). Sequences of both strands were determined.

Sequence alignments and phylogenetic trees. Sequences were edited with the Sequence Navigator software (PE Applied Biosystems). Multiple sequence alignments were performed with the Clustal X version 1.64 software (Higgins, Heidelberg, Germany). Trees and matrix distances were constructed with the Phylip

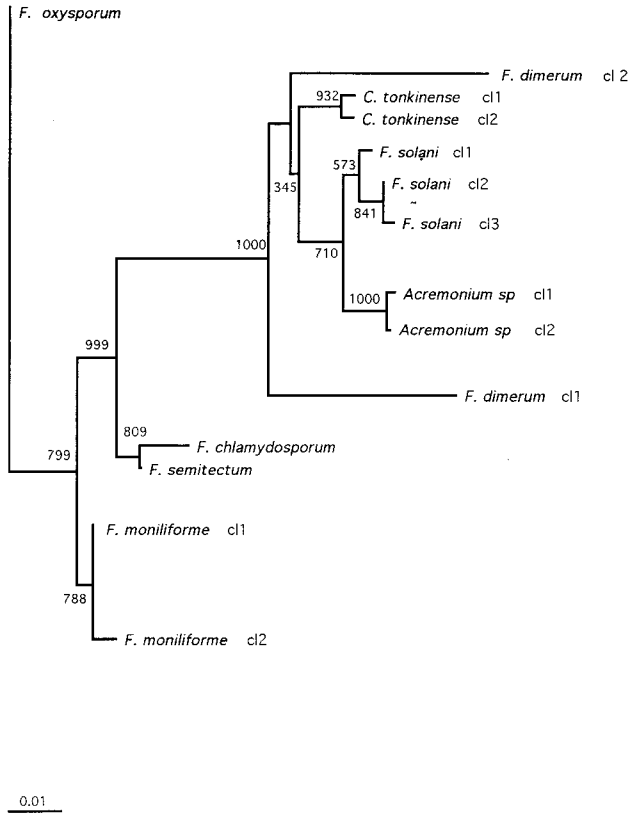

FIG. 1. Unrooted phylogenetic tree derived from 28S rDNA sequences of *Fusarium* spp., *C. tonkinense*, and *Acremonium* spp. The tree was constructed by using the neighbor-joining method. Bar, 1% estimated sequence divergence. cl, class.

package 3.572 by using the neighbor-joining method. The degree of confidence in phylogenetic branching was assessed by using 1,000 bootstrap resamplings.

Nucleotide sequence accession numbers. The nucleotide sequences of the strains and isolates tested in this work appear in the GenBank nucleotide sequence database under the accession numbers shown in Table 1.

RESULTS AND DISCUSSION

In this work, we compared the conventional methods for identification of *Fusarium* species with a 28S rDNA sequence method. Using the newly designed primers Fus1 and Fus2, we were able to amplify a 480-bp fragment from 33 *Fusarium* strains and isolates and from strains and isolates from the closely related genera *Acremonium* and *Cylindrocarpon*. In contrast, we failed to amplify the DNAs from nine reference strains belonging to the unrelated species listed in Table 1. Sequencing of the PCR products led to 10 sequence classes for *Fusarium* strains and isolates, each containing 1 to 10 isolates. Comparison with *Fusarium* sequences from the GenBank database demonstrates complete interspecies identity with those of *Fusarium semitectum* (accession no. X80813), *F. oxysporum* (U34537 and U34542), and *F. moniliforme* (U34528). The sequence of *F. dimerum* (U88105) exhibits close similarity with that of *F. dimerum* sequence class 2, with a difference of only 6 nucleotides (98.6% homology). Comparison of sequences for *F. solani* is possible only with sequences obtained from *F. solani* forma speciales *phaseoli* isolates (L36629, L36630, L36632, and L36634), which are involved in the soybean sudden death syndrome. They exhibit a closer homology with *C. tonkinense* sequence class 2 (98.16%) than with *F. solani* sequence class 1 (96.74%). Multiple alignment of our sequence classes was carried out by using Clustal X software (15). These data were used to construct an unrooted phylogenetic tree by the neighbor-joining method (12). As illustrated in Fig. 1, each *Fusarium* species as determined by conventional methods formed a distinct clade, with a minimum bootstrap confidence value of 71% for *F. solani* (Fig. 1). Interspecies homologies for *Fusarium* never reached higher than 99.01% (i.e., for *Fusarium chlamydosporum* and *F. semitectum*) (Fig. 2). In contrast, with the exception of *F. dimerum* (discussed below), intraspecies homologies ranged from 99.09% for *F. solani* to 100% for *F. oxysporum*. An isolate thus can be readily assigned to one species when its homology with one of the sequence classes is higher than 99.1%.

The isolate IHEM 5322, considered *F. dimerum*, exhibited a unique sequence, with 8.25% divergence from the other sequence class containing three strains of *F. dimerum*. Interest-

	F. oxysporum	*F. moniliforme* cl1	*F. moniliforme* cl2	*F. solani* cl1	*F. solani* cl2	*F. solani* cl3	*F. dimerum* cl1	*F. dimerum* cl2	*F. chlamydosporum*	*F. semitectum*	*Acremonium sp* cl1	*Acremonium sp* cl2	*C. tonkinense* sp cl1	*C. tonkinense* sp cl2
F. oxysporum	100													
F. moniliforme cl1	98.39	100												
F. moniliforme cl2	97.92	99.54	100											
F. solani 1	92.83	93.59	93.08	100										
F. solani 2	92.58	93.35	92.83	99.32	100									
F. solani 3	92.32	93.09	92.57	99.09	99.77	100								
F. dimerum cl1	91.57	91.54	91.01	95.52	95.28	95.03	100							
F. dimerum cl2	90.41	91.21	90.67	94.25	94.25	94.51	91.75	100						
F. chlamydosporum	96.27	97.46	93.83	93.83	93.08	93.82	92.02	91.7	100					
F. semitectum	94.81	98.39	94.31	94.06	94.07	93.81	92.28	94.81	99.09	100				
Acremonium sp cl1	92.34	93.59	93.08	98.16	98.39	98.62	93.81	94.01	93.82	94.31	100			
Acremonium sp cl2	92.32	93.34	92.82	98.16	98.39	98.62	93.8	94	93.57	94.06	99.77	100		
C. tonkinense cl1	93.34	94.09	93.58	98.16	97.45	97.22	94.03	95	93.83	94.81	96.26	96.26	100	
C. tonkinense cl2	93.34	94.09	93.58	98.62	97.93	97.69	94.54	94.75	93.83	94.81	96.75	96.74	99.54	100

FIG. 2. Homology between *Fusarium* spp., *Acremonium* spp., and *C. tonkinense*, derived from 28S rDNA sequences. The sequence classes (cl) correspond to those in Table 1.

TABLE 1. Strains and isolates tested for 28S rDNA sequences in this study

Strain category	Species	Strain(s)[a]	28S rDNA sequence class	Accession no.
Reference strains	*Acremonium killiense*	IP 1577	*Acremonium* class 2	AF130374
	Aspergillus flavus	IP 954		
	Aspergillus fumigatus	IP 1079		
	Candida albicans	ATCC 90028		
	Candida glabrata	ATCC 90030		
	Candida parapsilosis	ATCC 90018		
	Cryptococcus neoformans	ATCC 90112		
	Cylindrocarpon tonkinense	IP 1692	*C. tonkinense* class 1	AF130375
		IP 2329	*C. tonkinense* class 2	AF130376
	Fusarium chlamydosporum	IP 1542	*F. chlamydosporum*	AF130377
	Fusarium dimerum	IP 1516, IP 2365	*F. dimerum* class 1	AF130378
		IHEM 5322	*F. dimerum* class 2	AF130379
	Fusarium moniliforme	IHEM 3824, IHEM 7465, IP 1550	*F. moniliforme* class 1	AF130384
		IHEM 4195, IP 1579	*F. moniliforme* class 2	AF130385
	Fusarium oxysporum	IHEM 13830, IHEM 3798, IHEM 9571, IP 625	*F. oxysporum*	AF130373
	Fusarium semitectum	IP 2239	*F. semitectum*	AF130380
	Fusarium solani	IP 1684	*F. solani* class 1	AF130381
		IHEM 10154	*F. solani* class 2	AF130382
		IHEM 6092, IP 2330	*F. solani* class 3	AF130383
	Paecilomyces variotti	ATCC 22319		
	Scedosporium apiospermum	IP 1698		
	Scedosporium prolificans	IP 1974		
Clinical isolates	*Acremonium* sp.	N97110423	*Acremonium* sp. class 1	AF130386
		N97128916, N96114830	*Acremonium* sp. class 2	
	F. dimerum	HM94.1	*F. dimerum* class 1	
	F. oxysporum	GEMO9795,[b] GEMO9796,[b] GEMO9896, N95151312, N96148518, N98117566	*F. oxysporum*	
	F. solani	C98.1	*F. solani* class 1	
		HM94.2, N96144236[c]	*F. solani* class 3	
	F. moniliforme	GEMO9908,[d] N98119873, N96146918, N96149460, PS97.1	*F. moniliforme* class 1	

[a] IP, Pasteur Institute collection; IHEM, Institute of Hygiene and Epidemiology Mycology; ATCC, American Type Culture Collection.
[b] First identified as *F. solani*.
[c] Not pigmented isolate.
[d] First identified as a *Fusarium* sp.

ingly, this isolate had deletions at positions 398 and 403, a characteristic shared only with the other sequence class corresponding to *F. dimerum*. We have no explanation for this discrepancy. (It should be mentioned that this strain had been collected not from a patient but from the recycled water of a spray humidifier.) Such a discrepancy between a molecular method (in this case using species-specific probes) and the conventional method has already been described for a *Fusarium* reference strain (3), suggesting that conventional methods may not be accurate for some strains with atypical phenotypic characteristics.

While the aim of this work was not a phylogenic reevaluation, we noted that there are closer relationships between some genera tested in this study than between some species within the *Fusarium* genus. This is illustrated by the grouping of *F. solani*, *Acremonium* spp., and *C. tonkinense* (intersequence class divergences lower than 6.24%), whereas *F. moniliforme* and *F. oxysporum* exhibited mean divergences from *F. solani* between 6.98 and 7.42%, respectively. Interestingly, *F. solani*, *Acremonium* spp., and *Cylindrocarpon* are characterized by teleomorphs belonging to the genus *Nectria*, whereas *F. moniliforme* and *F. oxysporum* have teleomorphs belonging to the genus *Giberella*, reflecting the heterogeneity of the genus *Fusarium* (5, 10, 11).

For diagnosis purposes, the sequencing method showed its objective value by easily and correctly identifying a nonpigmented *F. solani* isolate (N96144236), independent of the mycologist's experience. Also, isolates with uncertain or wrong conventional identifications were identified in agreement with the Institute of Hygiene and Epidemiology Mycology reference center identification. Finally, the sequencing method allows identification within 48 to 72 h following a 3-day culture of the isolate, as opposed to the time-consuming conventional methods. Recently, an exoantigen test was proposed for the species identification of *Fusarium*, which requires the production of antiserum against specific antigens (6 weeks of incubation) and the extraction of exoantigens from 10-day-old cultures (14). Our results demonstrate that 28S rDNA sequencing is a valuable method for the identification of significant *Fusarium* species involved in human infections.

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