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Ribosomal DNA Internal Transcribed Spacer Analysis Supports Synonomy of *Scedosporium inflatum* and *Lomentospora prolificans*

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***Scedosporium inflatum* is a dematiaceous opportunistic pathogen originally described by D. Malloch and I. F. Salkin (Mycotaxon 21:247–255, 1984). However, E. Gueho and G. S. De Hoog (J. Mycol. Med. 118:3–9, 1991) recently suggested reducing this mold to synonomy with *Lomentospora prolificans* on the basis of their similar morphological and molecular characteristics. We have investigated the ribosomal DNA internal transcribed spacers (ITS), i.e., ITS I and ITS II, of 18 isolates, including these two fungi and a closely related pathogen, *Scedosporium apiospermum*, and its telemorph, *Pseudallescheria boydii*. Identical ITS restriction fragment length polymorphisms were found in eight isolates of *S. inflatum* and *L. prolificans*. These results support Gueho and De Hoog's proposal to combine *S. inflatum* and *L. prolificans* into the binomial *Scedosporium prolificans*. However, the ITS I sequence of *S. apiospermum* and the ITS restriction fragment length polymorphisms of *S. apiospermum* and *P. boydii* were found to be significantly different from those of *S. inflatum* and *L. prolificans*. The ITS restriction pattern differences may be valuable in clinical settings for distinguishing these fungi.**

Scedosporium inflatum Malloch et Salkin, a dematiaceous hyphomycete, is an important emerging human pathogen which has been associated with subcutaneous and systemic infections in immunocompromised patients (12, 14–16, 22). Since its morphologic and physiologic characteristics are similar to those of several other dark molds, *S. inflatum* has occasionally been misidentified for other fungal pathogens such as *Wardomyces humicola*, *Lomentospora prolificans*, and *Scedosporium apiospermum*. Gueho and De Hoog (12) recently suggested, on the basis of ultrastructure studies and DNA-DNA hybridization analysis, that *S. inflatum* and *L. prolificans* were conspecific and proposed the binomial *Scedosporium prolificans*.

DNA sequence analysis (1–3, 7, 13) has been used as a new approach to differentiate fungal pathogens. Internal transcribed spacers (ITS) between the 18S, 5.8S, and 28S ribosomal genes are useful regions of the fungal genome for determining species-specific and variety-specific relationships of fungi (4, 10, 13). Because of the high mutation rates of these regions, they are not highly conserved. In addition, these regions can be easily amplified by the PCR (21). In this study, we amplified and analyzed the ITS of *S. inflatum*, *L. prolificans*, and *S. apiospermum* and its telemorph, *Pseudellascheria boydii*, to evaluate Gueho and De Hoog's taxonomic proposal and the application of DNA sequence analysis to differentiate closely related fungal pathogens.

MATERIALS AND METHODS

Isolates. The isolates used in this study are listed in Table 1. Isolates were maintained on Sabouraud dextrose agar (Difco

TABLE 1. Isolates used in this study

Isolate[a]	Accession no.[b]	Source
Group I		
S. inflatum	M236-92	Skin lesions, blood
	M269-92 (type), CBS 114.90	Bone from foot
	NCMH 2365	Osteomyelitis
	NCMH 2708	Peritoneal cavity
	NCMH 2850	Right foot
	NCMH 2705	Sputum
L. prolificans	M270-92 (type), CBS 467.74	Greenhouse soil
	M352-92	—[c]
Group II		
S. apiospermum	M356-89	Anterior ear
	M895-90	Brachiotracheotomy
	M227-93	Face wound
	M426-92	Middle finger
	M1175-91	Left ear
P. boydii	M128-90	Skin lesion
	NUS20P	—
	M472-78	Bronchial wash
	M1574-85	Heart valve
	M76-90	Fourth finger

[a] Group I isolates are those involved in the question of the synonomy of *S. inflatum* and *L. prolificans*. Group II isolates are those hard to distinguish clinically from *S. inflatum* and *L. prolificans*.

[b] M, NCMH, and NU-designated isolates were all obtained from the Laboratories of Mycology, Wadsworth Center Laboratories and Research, New York State Department of Health, Albany, N. Y. CBS, Centralbureau voor Schimmelcultures, Baarn, The Netherlands.

[c] —, information not available.

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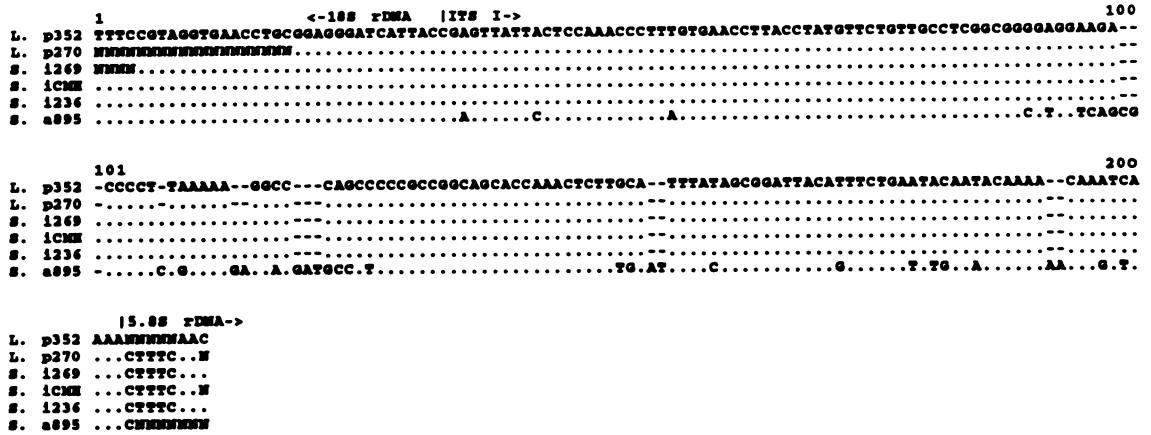

FIG. 1. ITS I sequence data for *S. inflatum*, *L. prolificans*, and *S. apiospermum*. Abbreviations: L. p352, *L. prolificans* M352; L. p270, *L. prolificans* M270; S. i269, *S. inflatum* M269; S. iCMH, *S. inflatum* NCMH 2365; S. i236, *S. inflatum* M236; S. a895, *S. apiospermum* M895; N, unknown nucleotide base; ., nucleotide base that is identical to the one on the top line; -, gap introduced for alignment purposes.

Laboratories, Detroit, Mich.) slants at 17°C, due to incubator availability, prior to sampling for DNA extraction.

DNA extraction. DNA was extracted from the mycelium by the method described by Goodwin and Lee (11), with modifications. Samples (approximately 0.5 cm^2) were taken from culture slants and placed into individual microcentrifuge tubes containing 50 μl of lysis buffer (50 mM Tris-HCl [pH 7.2], 50 mM EDTA, 3% sodium dodecyl sulfate, 1% 2-mercaptoethanol). The tubes were placed into a 400-W, common microwave oven (Little Litton) and microwaved, with lids opened and overlaid with cellophane wrap, three times for 10 s each time. The microwave oven is used to aid in cell wall and cell membrane alteration. Following the microwaving of the tubes, 350 μl of additional lysis buffer was added to each microcentrifuge tube, and the tubes were then incubated at 80°C for 5 min. After incubation of the tubes, 400 μl of 1:1 chloroform-phenol was added to each tube, and following the vortexing of the tubes, they were centrifuged at 10,000 × *g* for 5 min. The aqueous phase (top layer) was removed and placed into a new tube containing 0.54 volume of isopropanol and 10 μl of 5 M sodium acetate. These tubes were centrifuged at 10,000 × *g* for 2 min, and the supernatant was decanted. The remaining pellet was washed with 80% ethanol and, depending upon the size of the pellet, resuspended in 30 to 50 μl of Millipore-filtered H_2O (filter pore size, 0.45 μm). DNA concentrations were estimated by electrophoresing 3 μl of the samples in 0.8% agarose gels beside lambda DNA standard (1.0 μg/μl).

PCR amplification. Ribosomal ITS I and II and the 5.8 S rDNA coding for rRNA (rDNA) were amplified by the PCR method, with ITS 1 and ITS 4 used as primers (21), 0.1 to 1.0 ng of total genomic DNA used as the template, and thermal cycling performed with an automated Perkin-Elmer cycler (model 480; Perkin-Elmer Cetus, Norwalk, Conn.), as described by White et al. (21). Controls without the DNA template were used with the amplification mixtures to test for contaminating DNA.

Direct sequencing of symmetric and asymmetric PCR products. For direct DNA sequencing, double-stranded templates generated by symmetric PCR (with equal amounts of primers) as well as single-stranded templates generated by asymmetric PCR (in which one primer is in excess of the other in a 50:2.5 ratio) representing ITS I, ITS II, and 5.8S rDNA were sequenced with external primers ITS 1 and ITS 4 and internal primers ITS 2 and ITS 3 (21). Taquence ^{35}S DNA sequencing kits (U.S. Biochemical Corp., Cleveland, Ohio) were used for the dideoxynucleotide chain-terminating DNA sequencing method of Sanger et al. (17), as modified by Brow (5) and more recently described by Lee and Taylor (13). Sequencing with

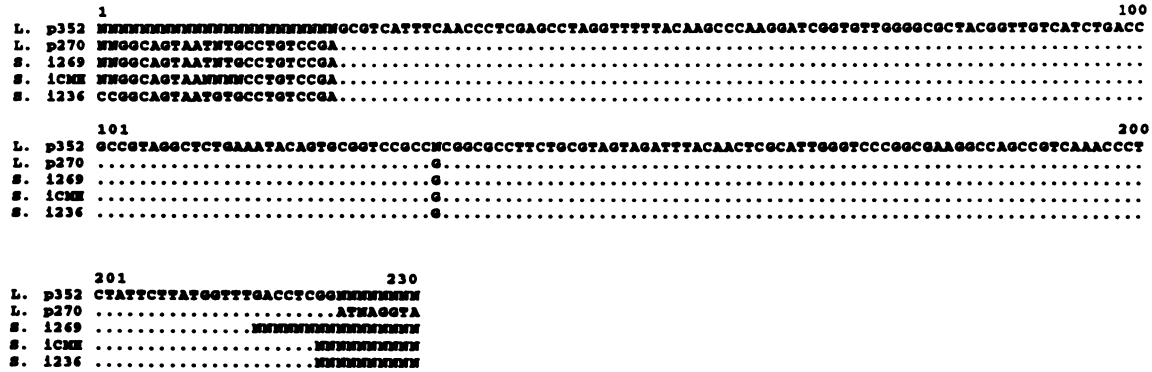

FIG. 2. ITS II sequence data for *S. inflatum* and *L. prolificans*. Abbreviations: L. p352, *L. prolificans* M352; L. p270, *L. prolificans* M270; S. i269, *S. inflatum* M269; S. iCMH, *S. inflatum* NCMH 2365; S. i236, *S. inflatum* M236; N, unknown nucleotide base; ., nucleotide base that is identical to the one on the top line; -, gap introduced for alignment purposes.

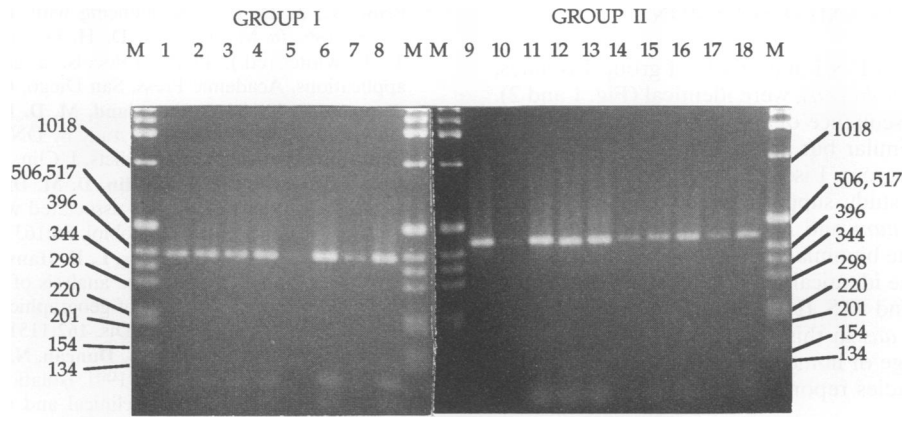

FIG. 3. *Hae*III digestion of PCR products generated from isolates of group I (*S. inflatum* and *L. prolificans*) and group II (*S. apiospermum* and *P. boydii*) by use of primers ITS 1 and ITS 4. Lane 1, *S. inflatum* M236; lane 2, *S. inflatum* M269; lane 3, *S. inflatum* NCMH 2365; lane 4, *L. prolificans* M270; lane 5, *L. prolificans* M352; lane 6, *S. inflatum* NCMH 2708; lane 7, *S. inflatum* NCMH 2850; lane 8, *S. inflatum* NCMH 2705; lane 9, *S. apiospermum* M356; lane 10, *S. apiospermum* M895; lane 11, *P. boydii* M128; lane 12, *P. boydii* NUS20P; lane 13, *P. boydii* M472; lane 14, *P. boydii* M1574; lane 15, *P. boydii* M76; lane 16, *S. apiospermum* M227; lane 17, *S. apiospermum* M426; lane 18, *S. apiospermum* M1175. A 1-kb DNA ladder marker (lanes M) was used to flank the samples; sizes (in base pairs) are on both sides of the figure.

both internal and external primers enabled the sequencing of ITS I and ITS II of *L. prolificans* and *S. inflatum* in both directions.

Sequencing samples were run at 1,500 V on 5% acrylamide wedge gels at a constant temperature of 50°C. DNA sequences were read from autoradiographs developed after a 12- to 72-h exposure of Kodak X-Omat film to the dried gels. DNA sequence data were directly transferred to a personal computer (IBM compatible) with an automated gel reader (Compu-Gene, Chesterfield, Mo.). Sequences were aligned by the alignment subroutine GENALIGN (Intelligenetics, Mountain View, Calif.). The combined ITS I and ITS II data set had approximately 430 aligned nucleotide positions, and some were scored as unknowns.

Restriction endonuclease digestion and gel electrophoresis. The restriction endonucleases *Hae*III and *Hha*I (Gibco BRL/Life Technologies, Gaithersburg, Md.) were used in separate digestion reactions with PCR-amplified ITS fragments from the isolates listed in Table 1. The digestions were performed in 1.5-ml microcentrifuge tubes with 8 μl of PCR product, 8 μl of distilled H_2O, 2 μl of restriction endonuclease, and 2 μl of enzyme buffer (provided by the manufacturer). Digests were incubated for 2 h at 37°C to allow the reactions to proceed to completion.

Restriction fragment length polymorphism (RFLP) analysis was performed by loading 10 μl of the *Hae*III- and *Hha*I-generated ITS fragments onto 2% NuSieve–1% agarose (FMC BioProducts, Rockland, Maine) gels in 1× TAE buffer (0.1 M Tris-HCl, 12.5 mM sodium acetate, 1 mM EDTA [pH 8.1]) and electrophoresing for approximately 2 h at 90 V. The gels were stained in ethidium bromide (0.5 μg/ml) for 30 min, destained for 30 min in distilled water, and then photographed under UV light.

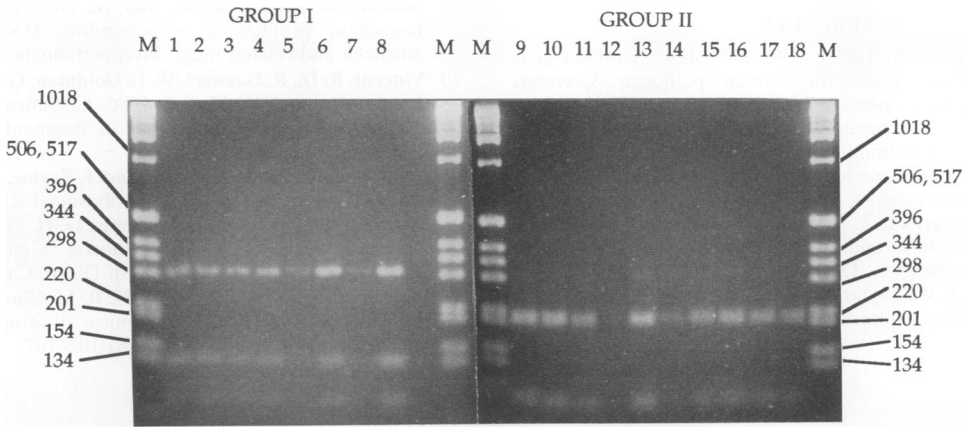

FIG. 4. *Hha*I digestion of PCR products generated from isolates of group I (*S. inflatum* and *L. prolificans*) and group II (*S. apiospermum* and *P. boydii*) by use of primers ITS 1 and ITS 4. Lane 1, *S. inflatum* M236; lane 2, *S. inflatum* M269; lane 3, *S. inflatum* NCMH 2365; lane 4, *L. prolificans* M270; lane 5, *L. prolificans* M352; lane 6, *S. inflatum* NCMH 2708; lane 7, *S. inflatum* NCMH 2850; lane 8, *S. inflatum* NCMH 2705; lane 9, *S. apiospermum* M356; lane 10, *S. apiospermum* M895; lane 11, *P. boydii* M128; lane 12, *P. boydii* NUS20P; lane 13, *P. boydii* M472; lane 14, *P. boydii* M1574; lane 15, *P. boydii* M76; lane 16, *S. apiospermum* M227; lane 17, *S. apiospermum* M426; lane 18, *S. apiospermum* M1175. A 1-kb DNA ladder marker (lanes M) was used to flank the samples; sizes (in base pairs) are on both sides of the figure.

RESULTS AND DISCUSSION

The gene sequences in ITS I and ITS II of group I isolates, i.e., *S. inflatum* and *L. prolificans*, were identical (Fig. 1 and 2). In contrast, the ITS I sequence of a group II isolate, *S. apiospermum* M895, was similar but approximately 17% different from the sequences of group I isolates (Fig. 1).

The results of this study support Gueho and De Hoog's conclusion that *S. inflatum* and *L. prolificans* are conspecific and that the appropriate binomial for this opportunistic pathogen is *S. prolificans*. The identical DNA sequences from highly nonconserved ITS I and ITS II found among isolates of *S. inflatum* and *L. prolificans* in this investigation are consistent with the high percentage of homology demonstrated in DNA-DNA hybridization studies reported by Gueho and De Hoog (12).

RFLP patterns of ITS I, ITS II, and 5.8S rDNA of group I isolates were identical (Fig. 3 and 4). RFLP patterns of ITS I, ITS II, and 5.8S rDNA of group II isolates were also identical (Fig. 3 and 4) but significantly different from the RFLP patterns of group I isolates.

Similar RFLP techniques have been applied in studies using total genomic DNA from *Candida albicans* (6) and *Aspergillus fumigatus* (8), using mitochondrial DNA of *Sporothrix schenckii* (9, 19), and using PCR-generated rDNA of *Histoplasma capsulatum* (18, 20). Such studies have been successful in providing discriminating patterns for epidemiological purposes. However, restriction analysis of genomic and mitochrondrial DNA requires a greater fungal biomass than PCR-based restriction fragment methods.

We have demonstrated in this investigation that PCR-based restriction fragment methods can be used to differentiate closely related pathogens; i.e., group I (*S. inflatum* and *L. prolificans*) isolates can be distinguished from group II (*S. apiospermum* and *P. boydii*) isolates. As noted, since only limited fungal biomass is needed for a PCR-based technique, the small concentration of fungi generally present in clinical specimens should provide adequate amounts of starting templates for detecting and identifying these pathogens.

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