

Prevalence and Susceptibility Testing of New Species of *Pseudallescheria* and *Scedosporium* in a Collection of Clinical Mold Isolates[∇]

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The prevalence of new species of *Pseudallescheria* and *Scedosporium* in a collection of 46 clinical isolates was analyzed. Strain identification was done by morphological and molecular methods. Four *Scedosporium aurantiacum* isolates were detected among the panel of clinical strains. The susceptibility profile of *S. aurantiacum* was similar to that of *Scedosporium apiospermum*.

Pseudallescheria boydii is a pathogen able to cause asymptomatic colonization and localized and disseminated infections (11).

Recently, it has been demonstrated that high genetic variation exists in the *P. boydii* species complex (2, 4, 8). Two new species, *Pseudallescheria minutispora* and *Scedosporium aurantiacum*, are phylogenetically and morphologically separated from *P. boydii*. In addition, *P. angusta*, *P. ellipsoidea*, and *P. fusioidea* seem to present genetic differences and could be proposed as new species of *Pseudallescheria* (4).

We have analyzed the prevalence of these new species and their antifungal susceptibility profiles in a collection of clinical isolates of *P. boydii*.

Strains. A total of 46 clinical isolates of *P. boydii* and *S. apiospermum* were included in this study. Twenty-four strains were isolated from respiratory sites, five from biopsies, six from ear samples, five from skin, four from ocular samples, one from blood culture, and one from an abscess.

Table 1 displays the identification of the 42 sequences obtained from the GenBank database that were used as controls.

Morphological identification. All isolates were identified by conventional methods (3).

PCR and DNA sequencing of the internal transcribed spacer (ITS) region. Molds were cultured in GYEP medium (0.3% yeast extract, 1% peptone [Difco, Madrid, Spain], 2% glucose [Sigma Aldrich Quimica, Madrid, Spain]) for 24 to 48 h at 30°C. Genomic DNA was isolated using a previously described extraction procedure (5).

DNA segments comprising the ITS1 and ITS2 regions were amplified with primers ITS1 (5'-TCCGTAGGTGAACCTGGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (1) in a GeneAmp 9700 PCR system (Applied Biosystems) (10). The reaction products were analyzed in a 0.8% agarose gel.

Sequencing reactions were done with 2 µl of reaction mixture from a sequencing kit (BigDye terminator cycle sequencing kit, Ready Reaction mixture; Applied Biosystems), 1 µM of

the primers (ITS1 and ITS4), and 3 µl of the PCR product in a final volume of 10 µl.

Sequence analysis. Sequences were assembled and edited using the SeqMan II and EditSeq software packages (LaserGene; DNASTar, Inc., Madison, WI). Sequence analysis was performed by comparison of the DNA sequences with

TABLE 1. *Scedosporium* reference strains used for comparison of ITS sequences and their GenBank accession numbers

Species	Strain identification	GenBank accession no.
<i>Pseudallescheria angusta</i>		AJ888414
<i>Pseudallescheria angusta</i>		AJ888413
<i>Pseudallescheria ellipsoidea</i>	CBS418,73	AJ888426
<i>Pseudallescheria ellipsoidea</i>		AJ888427
<i>Pseudallescheria fusioidea</i>	CBS106,53	AJ888428
<i>Pseudallescheria fusioidea</i>		AJ888429
<i>Pseudallescheria minutispora</i>		AJ888424
<i>Pseudallescheria minutispora</i>		AY228119
<i>Pseudallescheria minutispora</i>		AJ888384
<i>Scedosporium apiospermum</i>		AJ888443
<i>Scedosporium apiospermum</i>		AJ888392
<i>Scedosporium apiospermum</i>		AF181558
<i>Scedosporium apiospermum</i>		AJ888385
<i>Scedosporium apiospermum</i>		AJ888438
<i>Scedosporium apiospermum</i>		AJ888391
<i>Scedosporium apiospermum</i>		AY213683
<i>Scedosporium apiospermum</i>	CBS 10854	AY228112
<i>Scedosporium apiospermum</i>		AJ888400
<i>Scedosporium apiospermum</i>		AY217658
<i>Scedosporium apiospermum</i>		AY228123
<i>Scedosporium apiospermum</i>		AY939802
<i>Scedosporium apiospermum</i>		AF455484
<i>Scedosporium apiospermum</i>	CBS 10122	AY213680
<i>Scedosporium apiospermum</i>		AY213681
<i>Scedosporium apiospermum</i>	CBS 59190	AY228118
<i>Scedosporium apiospermum</i>		AY228122
<i>Scedosporium apiospermum</i>		AY213682
<i>Scedosporium apiospermum</i>		AJ888398
<i>Scedosporium apiospermum</i>		AJ888436
<i>Scedosporium apiospermum</i>		AJ888386
<i>Scedosporium apiospermum</i>		AJ888387
<i>Scedosporium apiospermum</i>		AJ888394
<i>Scedosporium apiospermum</i>		AJ888397
<i>Scedosporium apiospermum</i>		AJ888405
<i>Scedosporium apiospermum</i>		AJ888410
<i>Scedosporium apiospermum</i>		AJ888411
<i>Scedosporium apiospermum</i>	CBS101,22	AJ888435
<i>Scedosporium apiospermum</i>		AJ888437
<i>Scedosporium aurantiacum</i>		AJ888432
<i>Scedosporium aurantiacum</i>		AJ888439
<i>Scedosporium aurantiacum</i>		AJ888440
<i>Scedosporium aurantiacum</i>		AJ888441

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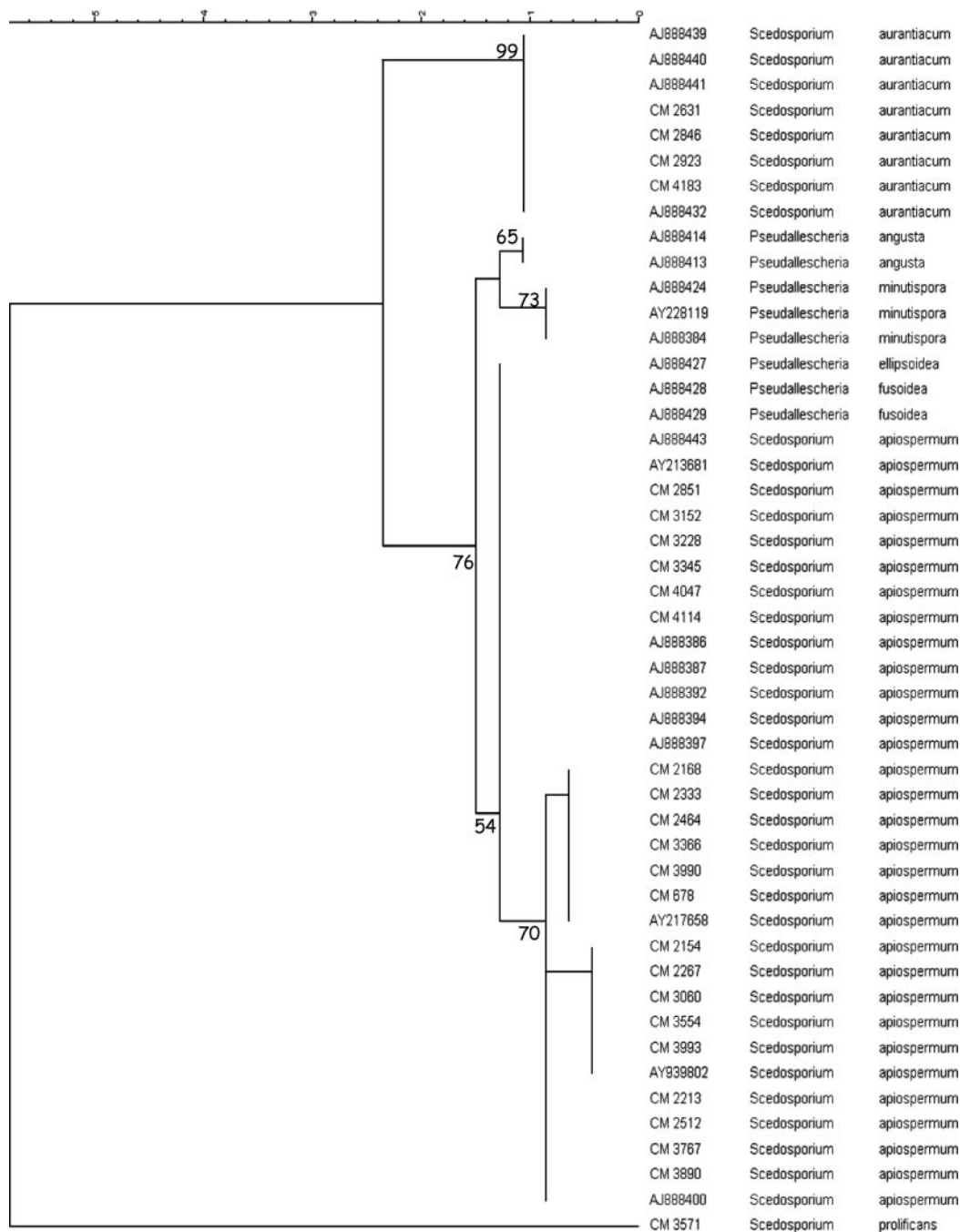


FIG. 1. Phylogenetic tree of the subset of isolates included in the study obtained by using maximum parsimony phylogenetic analyses and 2,000 bootstrap simulations based on ITS sequences. *Scedosporium prolificans* CNM-CM-3751 was used as the outgroup to root the tree.

42 ITS sequences of *Scedosporium* and *Pseudallescheria* strains obtained from the GenBank database (<http://www.ncbi.nih.gov/GenBank/>). Full information about these strains is displayed in Table 1.

Phylogenetic analysis. All phylogenetic analyses were conducted with Fingerprinting II Informatix software, version 3.0 (Bio-Rad Laboratories, Madrid, Spain). The methodology used was maximum parsimony clustering. Phylogram stability was assessed by parsimony bootstrapping with 2,000

simulations. The ITS sequence of *Scedosporium prolificans* CNM-CM-3571 (Mold Collection of the Spanish National Center for Microbiology) was used as the outgroup.

Antifungal susceptibility testing. Microdilution testing was performed following the CLSI (formerly NCCLS) reference method (6) with the following minor modifications. (i) RPMI 1640 was supplemented with glucose to reach a 2% concentration. (ii) Inoculum size was between 1×10^5 to 5×10^5 CFU/ml. Inoculum preparations were performed by means of

TABLE 2. Antifungal susceptibility results for clinical isolates of *Scedosporium aurantiacum* and geometric mean and range of the 42 *Scedosporium apiospermum* isolates

Isolate	Strain no.	Sample	MIC (mg/liter)		
			Amphotericin B	Itraconazole	Voriconazole
<i>S. aurantiacum</i>	CNM-CM-2923	Corneal	8	16	16
	CNM-CM-2631	Sputum	4	2	0.5
	CNM-CM-2846	Ear swab	16	8	1
	CNM-CM-4183	Bronchial aspirate	8	16	0.5
Geometric mean			6.96	6.06	1.15
<i>S. apiospermum</i>	Geometric mean		6.21	2.62	0.73
	Range		0.25–32	0.25–16	0.125–16

counting spores in a hemacytometer (1, 7, 9). *Aspergillus fumigatus* ATCC 2004305 and *Aspergillus flavus* ATCC 2004304 were used as quality control strains (6).

The antifungal agents used in the study were amphotericin B (range, 16 to 0.03 mg/liter) (Sigma Aldrich Química), itraconazole (range, 8 to 0.015 mg/liter) (Janssen S.A., Madrid, Spain), and voriconazole (range, 8 to 0.015 mg/liter) (Pfizer S.A.). The endpoint was the antifungal concentration that produced a complete inhibition of visual growth at 48 h.

Morphological identification. All strains were identified as *Scedosporium apiospermum* by conventional methods (3). We were not able to identify by morphological characteristics the new proposed species (4, 8).

Molecular identification. Figure 1 shows the phylogenetic analysis by means of maximum parsimony of a subset of the strains included in the study. Among 46 clinical strains analyzed, four *S. aurantiacum* strains were identified with a bootstrap value of ≥ 99 . They were isolated from sputum, bronchial aspirate, a corneal sample, and an ear swab. In this collection of clinical isolates, we did not find any strain of *P. minutispora* or *P. angusta*, but the sequences obtained from GenBank were supported by bootstrap values of 73 and 65, respectively. On the other hand, the analyses of ITS sequences indicate that *Pseudallescheria ellipsoidea* and *P. fusoidea* are indistinguishable from *S. apiospermum* isolates (Fig. 1).

Antifungal susceptibility testing. The MICs of antifungal agents for the collection of clinical isolates are shown in Table 2. They are sorted by species identification by means of ITS sequencing.

It has always been suspected that *Pseudallescheria boydii* was a complex of species. Hitherto, morphological analyses of *Pseudallescheria boydii* isolates did not allow for a clear separation among different species. However, the analysis of DNA sequences has permitted the proposal of new species from *Pseudallescheria*, such as *S. aurantiacum*, *P. minutispora*, *P. angusta*, *P. ellipsoidea*, and *P. fusoidea* (4, 8).

We examined the prevalence of these new species in a collection of clinical isolates. ITS sequence analyses revealed the presence of four *S. aurantiacum* strains, obtained from an ocular sample, an ear swab, bronchial aspirate, and sputum, and no strains of *P. minutispora*. Although Gilgado et al. (4) have defined morphological characteristics to identify *S. aurantiacum* and *P. minutispora*, great experience in classical tax-

onomy is required to perform this task. Therefore, it is not expected that many clinical microbiology laboratories are able to identify those new species unless they use molecular methodology. Regarding susceptibility to antifungal drugs, *S. aurantiacum* seems slightly more resistant than *S. apiospermum* to amphotericin B and itraconazole, although a higher number of isolates should be analyzed before any conclusion is drawn (Table 2).

Because little is known about the prevalence of these new species and therefore there are no studies regarding epidemiology, pattern of disease, risk factors, antifungal susceptibility testing, etc., we strongly recommend sending all strains of *Scedosporium* species involved in human infections to reference laboratories where those isolates can be properly identified to the species level and antifungal susceptibility testing can be performed. In this way, the importance of these new species can be ascertained. Meanwhile, and especially for clinical use, it would be better to maintain the use of the *S. apiospermum* name. From a practical point of view and for clinical microbiology laboratories, we suggest performing antifungal susceptibility testing rather than applying molecular methods for the identification of these species.

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