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Scopulariopsis, a Poorly Known Opportunistic Fungus: Spectrum of Species in Clinical Samples and In Vitro Responses to Antifungal Drugs

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Ninety-nine isolates of clinical origin, tentatively identified as Scopulariopsis or Microascus, were morphologically and molecularly characterized by a combined analysis of the D1/D2 domains of the 28S rRNA gene and a fragment of the elongation factor 1-α gene (EF1-α) sequences. The most prevalent species was Scopulariopsis brevicaulis (49.4%), followed by Scopulariopsis gracilis (14.4%), Scopulariopsis brumptii (7.2%), Microascus cinereus (5.2%), the Scopulariopsis candida species complex (3.1%), and Microascus cirrospores (Z.1%). The most common anatomic sites of isolation were the respiratory tract (61.6%), superficial tissue (19.2%), and deep tissue or fluid samples (19.2%). The antifungal susceptibilities of the isolates to eight drugs were tested in vitro, with all the drugs generally showing poor activity.

The genus Scopulariopsis contains both hyaline and dematiaceous molds, and their teleomorphs are included in the genus Microascus (order Microascales). They are saprobes commonly isolated from soil, air, plant debris, paper, and moist indoor environments (1–3). Some species are known to be opportunistic pathogens, mainly causing superficial tissue infections, and they represent some of the principal causes of nondermatophytic onychomycoses (4, 5). Less common clinical manifestations include keratitis following eye trauma (6) and otomycosis (7). The fungi have also been involved in deep tissue infections, mainly in immunocompromised and occasionally in immunocompetent patients, causing, for example, pneumonia (8), endophthalmitis (9), subcutaneous and brain abscesses (10, 11), invasive sinusitis (12), parotitis (13), and endocarditis (14, 15). The most frequently reported species in all clinical presentations and anatomic locations is Scopulariopsis brevicaulis. Other less frequent species are Scopulariopsis aereum, Scopulariopsis brumptii, Scopulariopsis flava, Microascus niger, Microascus cinereus, Microascus cirrospores, Microascus manginii, and Microascus trigonosporus (5, 16–18).

Currently, there are close to 40 accepted species of Scopulariopsis and Microascus. For many of these species, the anamorph-teleomorph connection has already been established (1, 5, 16). However, the sexual states of some Scopulariopsis species are still unknown.

According to the new International Code of Nomenclature for fungi, algae, and plants, the dual nomenclature system that has been traditionally used for fungi, which includes both anamorph and teleomorph states, is no longer allowed and hence a unique name must be chosen (19). We judge that since the name Scopulariopsis has been used much more frequently in the literature, including in medical publications, this name should have priority over Microascus. However, since no formal proposal has yet been submitted, in the present paper we apply the traditional nomenclature for known Scopulariopsis and Microascus species.

Although the isolation of Scopulariopsis species from clinical specimens is relatively easy, as they grow well on routine laboratory media, it might be difficult to identify them morphologically down to the species level (18). Histopathology has limited significance in diagnostics since in tissue, the fungi show features similar to those of other more common pathogenic molds, such as Aspergillus or Fusarium species (18, 20). The sequencing of the ribosomal operon has been used for the identification of clinical strains of Scopulariopsis, although the results may not be reliable because of insufficient availability of reference sequences in the public databases (17, 18, 21, 22). Interestingly, due to the high genetic variability of the internal transcribed spacer (ITS) sequences found in a large set of Scopulariopsis strains isolated from cheese, Ropars et al. (23) used the combined analysis of partial sequences of the long subunit (LSU) rRNA gene, β-tubulin (TUB), and elongation factor 1-α (EF1-α) genes for the taxonomic circumscription of Scopulariopsis species and proposed the EF1-α gene to be the most phylogenetically informative genomic region for identifying Scopulariopsis species.

The high rates of resistance of these fungi to practically all currently used antifungal agents, including amphotericin B (AMB) and voriconazole (VRC), which are among the most commonly used drugs for the prophylaxis and first-line treatment of systemic mold infections, is significant. The appropriate therapy for Scopulariopsis infections has yet to be defined (22, 24). The effectiveness of AMB has been estimated to be only about 40% of successful treatments (24), which has resulted in high mortality.
rates and infection relapses (15, 20). In vitro antifungal susceptibility studies on these fungi are scarce and have involved mainly topical drugs. Several clinical reports have underlined the lack of correlation between in vitro susceptibility test results and clinical outcomes (21, 22, 25).

Because in most of the clinical reports of Scopulariopsis infections, morphological identification of the etiological agent has not been confirmed at the molecular level, the real prevalence of Scopulariopsis species in clinical samples, apart from those from S. brevicaulis, is unknown. We therefore studied a large set of clinical isolates, most of which were received at a mycology reference laboratory in the United States, in order to define the species spectrum and the relative frequencies of Scopulariopsis in clinical specimens. The in vitro antifungal susceptibilities of the most prevalent species were also determined.

MATERIALS AND METHODS

Fungal isolates and sequences. Ninety-nine clinical isolates received as Scopulariopsis or Microascus species were included in this study. In addition, 23 type and reference strains were studied. Five D1/D2 rRNA gene and six elongation factor 1-α gene (EF1-α) sequences retrieved from GenBank were also included in the phylogenetic analyses.

Morphological identification. The isolates were subcultured onto potato-dextrose agar (PDA) (Pronadisa, Spain), oatmeal agar (OA) (30 g of filtered oat flakes, 20 g of agar, 1 liter of distilled water), and potato–carrot agar (PCA) (20 g each of filtered potatoes and carrots, 20 g of agar, 1 liter of distilled water) up to 21 days at 25°C in darkness. The microscopic features were obtained from direct wet mounts and slide cultures on PDA, OA, or PCA, mounted in lactoc acid or lactophenol. All isolates were morphologically identified as per Morton and Smith (2), de Hoog et al. (5), OA, or PCA, mounted in lactoc acid or lactophenol. All isolates were morphologically identified as per Morton and Smith (2), de Hoog et al. (5), and Guarro et al. (26).

DNA extraction, amplification, and sequencing. Isolates were grown on YES agar (20 g of yeast extract, 150 g of sucrose, 20 g of agar, 1 liter of distilled water) for 5 days at 25°C. The total genomic DNA was extracted from agar cultures using the PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA), according to the manufacturer’s protocol. DNA was quantified using a Nanodrop 3000 (Thermo Scientific, Madrid, Spain).

To amplify a 440-bp fragment of the D1/D2 domains of the 28S rRNA gene and a 1,200-bp fragment of the EF1-α gene (EF1-α/H11021), respectively. The amplified products were purified with the Dye DeoxyTIP purification system (Sigma-Aldrich, St. Louis, MO, USA) and stored at −20°C until sequencing.

Sequencing was made in both directions with the same primer pair used for amplification at Macrogen Europe (Macrogen Inc., Amsterdam, The Netherlands). The consensus sequences were obtained using the SeqMan software version 7.0.0 (DNASTar Lasergene, Madison, WI, USA).

Molecular identification and phylogenetic analysis. Preliminary molecular identification of the isolates was performed using BLAST searches for both amplified fragments. Only the sequences of type or reference strains deposited in the GenBank/EMBL database were considered for identification purposes. A maximal level of identity (MLI) of ≥98% was considered to allow for a species-level identification. MLI values of <98% provided identification only at genus level.

Multiple sequence alignments were made in MEGA version 5.05 (29) using the ClustalW application (30) and manually refined under the same software platform. The ambiguous areas of the alignment were removed using the Gblocks server (http://molevol.cmima.csic.es /castresana/Gblocks_server.html) (31).

Concordance of the D1/D2 and EF1-α gene data sets was evaluated with the partition-homogeneity test implemented with PAUP+ version 4.0b10 (32). The selection of the best nucleotide substitution model (GTR + G + I) was made using jModelTest version 2.1.1 (33). Phylogenetic reconstruction of the combined data sets was made with the maximum likelihood (ML) analysis under MEGA version 5.05 using nearest-neighbor interchange (NNI) as a heuristic method for tree inference. Support for the internal branches was assessed by a search of 1,000 bootstrapped sets of data. A bootstrap support (BS) of ≥70 was considered significant. In addition, Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling was performed using MrBayes version 3.2.1 with two simultaneous runs for 6 million generations. Bayesian posterior probabilities (PP) were obtained from the 50% majority-rule consensus of trees sampled every 100 generations, after removing the first 25% of the resulting trees. A PP value of ≥0.95 was considered significant. The strains Petriella setifera CBS 437.75 and Parascopodium putredinis CBS 127.84 were used as outgroups.

Antifungal susceptibility testing. Antifungal susceptibility testing was performed according to CLSI document M38-A2 (34). The minimal effective concentration (MEC) was determined at 24 h for the echinocandins, and the MIC was determined at 48 h for the remaining drugs. The MIC was defined as the lowest concentration exhibiting 100% visual inhibition of growth for AMB, VRC, itraconazole (ITC), and posaconazole (PSC) and an 80% reduction in growth for terbinafine (TRB).

Nucleotide sequence accession numbers. The clinical isolates characterized in this study have been deposited in GenBank under accession numbers HG380346 through HG380499.

RESULTS

Of the 99 isolates studied, 97 were morphologically identified as members of the Scopulariopsis or Microascus genus. The remaining two isolates were identified as a Scedosporium sp. and Phialo- simplex caninus, respectively. The Scopulariopsis isolates were characterized by moderately fast growth and flat, velvety or powdery, white, tan, dark brown, gray, or black colonies. Microscopically, they showed hyaline or dematiaceous septate hyphae with cylindrical or flask-shaped conidigenous cells (annelides) formed singly or in small groups directly on the vegetative hyphae or on short and usually branched conidiophores. The conidia were one celled, hyaline, light green to dark brown, flat at the base, globose, limoniform or bullet-shaped with a smooth or rough surface, hydrophobic, and produced in long chains. When present, teleomorphs were characterized by dark, ostiolate, globose to pyriform perithecia with or without a neck, superficial or immersed in the agar. The asci were ovate and eavesmented and contained eight one-celled, straw-colored, asymmetrical, reniform, lunate, or triangular ascospores.

Figure 1 shows the phylogenetic tree inferred from the ML and Bayesian analyses of the concatenated EF1-α and D1/D2 sequences of a representative number of the clinical isolates tested in this study, including the type and reference strains of clinically relevant species of the genus. Of the 48 isolates identified as S. brevicaulis, 8 were randomly chosen for the phylogenetic analysis, since the isolates in this group showed a high sequence similarity (≥99.3%). In the tree, the clades are named according to the degree of similarity with the type or reference strains of known species. Twenty-three well-supported clades were formed, 14 of which corresponded with previously recognized species.

The S. gracilis clade grouped the type strain of this species together with 14 clinical isolates. All the isolates were characterized by the production of abundant conidia, usually from well-differentiated branched conidiophores. Ten of these isolates also developed ascocoma and ascospores morphologically very similar to those of M. cinereus. However, they can be distinguished from M. cinereus mainly by having lunate ascospores, measuring 4.3 to 6.5 by 2 to 4 μm, and by the presence of branched conidiophores.
The isolates belonging to the clade of *M. cinereus* sensu stricto showed ascospores of variable shape (reniform, broadly lunate, or triangular) and that were slightly smaller (4 to 5.5 by 2.5 to 4 μm), and the conidiophores were mostly simple, usually reduced to a single conidiogenous cell growing directly on the vegetative hyphae (Fig. 2).

Ten isolates that morphologically resembled *M. trigonosporus* were grouped in the clades *Scopulariopsis* sp. I, II, and VI, which...
were phylogenetically distant from the type strain of that species (CBS 218.31), i.e., they have 97.8%, 97.6%, and 98.0% sequence similarities, respectively.

The clades *Scopulariopsis* sp. III to V, VII, and VIII comprise 6 clinical isolates that were not morphologically similar or phylogenetically related to any known species.

A large clade, named the *S. candida* complex, includes the epitype of *S. candida*, a reference strain of *M. manginii*, three reference strains of *M. niger*, and three clinical isolates, two of which had been morphologically identified as *S. candida* and one as *M. niger*. Since the different species included in this group showed high sequence similarities (>98.7%) but exhibited different morphological characteristics, all the species included in this group were treated as a complex. The two clinical isolates grouped in the clade *Scopulariopsis* sp. IX were morphologically identified as *M. manginii* but proved to be phylogenetically distant from the reference strain of this species (MUCL 41467) and from the epitype of its anamorph *S. candida* (<97.9% and <98.2% sequence similarities, respectively).

Molecular identification showed that the most common species was *S. brevicaulis* (49.4%) followed by *S. gracilis* (14.4%), *S. brumptii* (7.2%), *M. cinereus* (5.2%), the *S. candida* species complex (3.1%), and *M. cirrosus* (2.1%). Table 1 summarizes the key morphological and molecular identifications at the species level was 67%. The remaining isolates were identified with confidence only at the genus level.

Most clinical isolates studied were of respiratory origin (61.6%), mainly obtained from bronchoalveolar lavage (BAL) fluid and sputum samples, followed by superficial tissue samples (19.2%) principally isolated from the nails and skin. The remaining 19.2% of isolates were from miscellaneous deep tissue or sterile fluid specimens (Table 2). *S. brevicaulis* was the most common species from all clinical origins. *S. gracilis* was most frequently isolated from BAL fluid and sputum samples, while *S. brumptii* and *M. cirrosus* were only recovered from lower respiratory tract samples.

The results of the antifungal susceptibility testing are summarized in Table 3. All antifungal drugs showed similar low activities. AMB showed an overall geometric mean MIC (GM) and MIC\textsubscript{90} of 16.9 \(\mu\)g/ml and 32.0 \(\mu\)g/ml, respectively. The activities of the azoles were similar for all the species tested; VRC and PSC displayed GM values of 16.4 \(\mu\)g/ml and 14.6 \(\mu\)g/ml, respectively. In contrast, ITC showed almost no activity, with an MIC\textsubscript{90} of \(\approx32\) \(\mu\)g/ml. The echinocandins had the highest activities, with overall GMS of 4.0 \(\mu\)g/ml, 3.7 \(\mu\)g/ml, and 1.1 \(\mu\)g/ml for anidulafungin (AFG), caspofungin (CFG), and micafungin (MFG), respectively. TRB also showed limited activity, with an overall GM and MIC\textsubscript{90} of 1.9 \(\mu\)g/ml and 8 \(\mu\)g/ml, respectively. *M. cirrosus* and the *S. candida* complex were the most susceptible species.

**DISCUSSION**

This study involved the highest number of isolates of *Scopulariopsis* and *Microascus* of clinical origin ever to be evaluated to date. In agreement with earlier reviews of clinical cases (17, 22), *S. brevicaulis* was the most commonly isolated species in our study. *S. gracilis* was the second most commonly isolated species in our study, which is interesting since this fungus has never been reported in human infections or isolated from clinical specimens. In numerous isolates of this species, a sexual state was present that morphologically resembled that of *M. cinereus*. The teleomorph of *S. gracilis* probably has never been described in the literature because it is often confused with *M. cinereus*. In a recent review of 33 human invasive infections from *Scopulariopsis* or *Microascus* species, *M. cinereus* was, after *S. brevicaulis*, the second most commonly isolated species (12%) (17). However, our study emphasized some difficulties in the morphological identification of this species, since almost a third of the isolates morphologically identified as *M. cinereus* were found after sequencing to be from *S. gracilis*. The inadequacy of the morphological criteria was also
observed for the 10 isolates listed as M. trigonosporus, as they finally were found to correspond to three distant and probably undescribed phylogenetic species. Clarification regarding the taxonomic status of the morphospecies *M. trigonosporus* is important because some invasive infections have been attributed to this species, e.g., a pneumonia in a bone marrow recipient (35) and an endocarditis case (36). Only in the latter case was the identity of the fungus verified by ITS sequencing.

Morphological identification of clinical isolates of *Scopulariopsis* and *Microascus* is still useful since the features of conidia and sexual reproductive structures are quite characteristic for this fungal group, allowing for accurate identification at least to the genus level. Molecular tools are widely used in clinical laboratories for identification of fungi (17), with rRNA genes being the most commonly used target. However, a thorough taxonomic molecular study is still lacking. *Scopulariopsis* isolates from clinical cases were identified mostly by performing a BLAST search in GenBank (17, 21, 22). Our study demonstrated that this approach is not very useful, mainly due to the lack of reference sequences for comparison. Furthermore, the D1/D2 region, the primary target used for species identification in clinical reports, shows a low interspecific variation in this fungal group. We observed that the isolates of *S. brumptii* and many *Microascus* spp., including the type strains of *M. trigonosporus*, *M. cinereus*, and *S. gracilis*, were erroneously identified by BLAST searches as *M. circosus*, with MLI values of ≥98. A clinical isolate previously reported as *M. cirrosus* (22) was reidentified here as *S. gracilis*.

Other loci, such as ITS, which is the most commonly sequenced DNA fragment for the identification of clinical molds, was difficult to amplify and too variable for phylogenetic studies in *Scopulariopsis* species, proposing the EF1-α gene to be a more reliable phylogenetic marker (23). We have obtained good resolution using a two-gene phylogeny (D1/D2 and EF1-α). Only one clade, the *S. candida* species complex, could not be clearly resolved with the sequence data used. Interestingly, several isolates involved in this study could not be assigned to any of the currently accepted species and apparently might represent new species. However, further phylogenetic studies testing more genetic markers and reference strains are needed to clarify the taxonomic positions of such isolates.

### Table 1: Key morphological features of the most commonly identified species in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>Color</th>
<th>Shape</th>
<th>Arrangement</th>
<th>Conidial cells</th>
<th>Conidia</th>
<th>Size (μm)</th>
<th>Ornamentation</th>
<th>Characteristics of Sexual Structures</th>
<th>Characteristics of Conidial Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Microascus cirrosus</em></td>
<td>Gray</td>
<td>Present</td>
<td>Broadly reniform</td>
<td>Bottle-shaped on poorly differentiated conidiophores</td>
<td>Ovate or clavate</td>
<td>4.5–6 by 2–4</td>
<td>Verrucose</td>
<td>Single or in groups (3–10)</td>
<td>Single or in groups (3–10)</td>
</tr>
<tr>
<td><em>Microascus cinereus</em></td>
<td>Brownish-gray</td>
<td>Present</td>
<td>Broadly reniform</td>
<td>Bottle-shaped on poorly differentiated conidiophores</td>
<td>Ovate or clavate</td>
<td>3.5–5 by 2–3.5</td>
<td>Smooth</td>
<td>Single or in groups (3–10)</td>
<td>Single or in groups (3–10)</td>
</tr>
<tr>
<td><em>Scopulariopsis brevicollis</em></td>
<td>Yellowish-brown</td>
<td>Present</td>
<td>Broadly reniform</td>
<td>Bottle-shaped on poorly differentiated conidiophores</td>
<td>Globular or Light brown</td>
<td>4.5–6 by 2.5–4</td>
<td>Verrucose</td>
<td>Single or in groups (3–10)</td>
<td>Single or in groups (3–10)</td>
</tr>
<tr>
<td><em>Scopulariopsis candida</em></td>
<td>Brown</td>
<td>Present</td>
<td>Broadly reniform</td>
<td>Bottle-shaped on poorly differentiated conidiophores</td>
<td>Ovate or clavate</td>
<td>3.5–5 by 2–3.5</td>
<td>Smooth</td>
<td>Single or in groups (3–10)</td>
<td>Single or in groups (3–10)</td>
</tr>
<tr>
<td><em>Scopulariopsis gracilis</em></td>
<td>Brown</td>
<td>Present</td>
<td>Broadly reniform</td>
<td>Bottle-shaped on poorly differentiated conidiophores</td>
<td>Ovate or clavate</td>
<td>3.5–5 by 2–3.5</td>
<td>Smooth</td>
<td>Single or in groups (3–10)</td>
<td>Single or in groups (3–10)</td>
</tr>
</tbody>
</table>

### Table 2: Anatomical sources of isolates of *Scopulariopsis* and *Microascus* spp. from clinical samples

<table>
<thead>
<tr>
<th>Species</th>
<th>Superficial tissue (No.)</th>
<th>Deep tissues/ fluids (No.)</th>
<th>Upper respiratory tract (No.)</th>
<th>Lower respiratory tract (No.)</th>
<th>Total no. (%) of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Scopulariopsis brevicollis</em></td>
<td>15</td>
<td>10</td>
<td>8</td>
<td>15</td>
<td>48 (49.4)</td>
</tr>
<tr>
<td><em>Scopulariopsis candida</em></td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>10</td>
<td>14 (14.4)</td>
</tr>
<tr>
<td><em>Scopulariopsis brumptii</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>7 (7.2)</td>
</tr>
<tr>
<td><em>Microascus cinereus</em></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>5 (5.2)</td>
</tr>
<tr>
<td><em>Scopulariopsis manginii</em></td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3 (3.1)</td>
</tr>
<tr>
<td><em>Microascus cirrosus</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2 (2.1)</td>
</tr>
<tr>
<td>Other species</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>11</td>
<td>18 (18.6)</td>
</tr>
</tbody>
</table>

Total no. (%) of isolates: 19 (19.2) 19 (19.2) 11 (11.1) 50 (50.5) 97 (100)

* Includes *Scopulariopsis asperula*, *S. candida*, *Scopulariopsis fusca*, *Microascus manginii*, and *Microascus niger.*
Interestingly, most *S. gracilis* and *M. cinereus* and all *S. brumptii* and *M. cirrosus* isolates tested in our study were from respiratory samples, which suggests a tendency toward localization at this anatomic site. Considering that most of the isolates from a respiratory origin (68%) have been obtained from BAL fluid samples and that most of the isolates from a respiratory origin (68%) have been obtained from BAL fluid samples, which suggests a tendency toward localization at this anatomic site. Considering that most of the isolates from a respiratory origin (68%) have been obtained from BAL fluid samples, which suggests a tendency toward localization at this anatomic site.

With the exception of the study by Aguilar et al. (24), the antifungal susceptibilities of *Scopulariopsis* and *Microascus* species have been evaluated mainly in *S. brevicaulis* (25, 37–39). Although our study included newer antifungals, the results generally agree with previous data, showing resistance to practically all the available antifungal drugs. Similar data were obtained with testing of other fungi also belonging to the *Microascales*, such as *Scedosporium* spp. (40). In our study, susceptibility to AMB was rarely observed, VRC and PSC showed moderate activities against only a few isolates, and ITC and TBF showed almost no activity. The echinocandins, especially MFG and AFG, demonstrated better in vitro activities than the azoles; however, a high number of resistant isolates were also detected. Since no treatment guidelines are available for this group of fungi, therapies for most reported clinical cases were based on previous experience with those used for *Aspergillus* or other clinically relevant molds. Although most clinical cases reported negative outcomes regardless of the type of antifungal treatment (20, 22), VRC has shown some clinical efficacy (11, 15). Our results showed similar in vitro activities for VRC and PSC, although there are no clinical reports using PSC. Echinocandins have only rarely been used to treat *Scopulariopsis* infections. Beltrame et al. (41) unsuccessfully used CFG after negative results with VRC in a case of fungal sinusitis caused by *S. acrnomium*. More recently, Iwen et al. (17) reported a negative outcome with a combination of liposomal AMB plus MFG against an invasive infection by *S. brevicaulis*.

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