Molecular Typing of Australian Scedosporium Isolates Showing Genetic Variability and Numerous S. aurantiacum

Laurence Delhaes,*1,2 Azian Harun,*†1 Sharon C.A. Chen,*1 Quoc Nguyen,‡1 Monica Slavin,§1 Christopher H. Heath,¶1 Krystyna Maszewska,* Catriona Halliday,* Vincent Robert,**1 Tania C. Sorrell,*† the Australian Scedosporium (AUSCEDO) Study Group,3 and Wieland Meyer*†1

One hundred clinical isolates from a prospective nationwide study of scedosporiosis in Australia (2003–2005) and 46 additional isolates were genotyped by internal transcribed spacer–restriction fragment length polymorphism (ITS-RFLP) analysis, ITS sequencing, and M13 PCR fingerprinting. ITS-RFLP and PCR fingerprinting identified 3 distinct genetic groups. The first group corresponded to Scedosporium prolificans (n = 83), and the other 2 comprised isolates previously identified as S. apiospermum: one of these corresponded to S. apiospermum (n = 33) and the other to the newly described species S. aurantiacum (n = 30). Intraspecies variation was highest for S. apiospermum (58%), followed by S. prolificans (45%) and S. aurantiacum (28%) as determined by PCR fingerprinting. ITS sequence variation of 2.2% was observed among S. apiospermum isolates. No correlation was found between genotype of strains and their geographic origin, body site from which they were cultured, or colonization versus invasive disease. Twelve S. prolificans isolates from 2 suspected case clusters were examined by amplified fragment length polymorphism analysis. No specific clusters were confirmed.

Despite efforts to identify and eliminate infectious agents, they continue to emerge and reemerge (1). Among them, pathogenic fungi contribute substantially to illness and death, especially in immunocompromised patients (2,3). In contrast to the well-documented opportunists Candida albicans, Cryptococcus neoforms, and

Aspergillus fumigatus, the epidemiology and evolution of human infections caused by uncommon but emerging fungi are incompletely understood. Such pathogens include Scedosporium apiospermum (teleomorph Pseudallescheria boydii) and S. prolificans, which are inherently resistant to many antifungal agents (3–5).

*These authors contributed equally to experimental work and data analysis.

1These current affiliation: Lille Pasteur Institute, Lille, France

2Members of the Australian Scedosporium Study Group of the Australasian Society for Infectious Diseases: Australian Capital Territory: Peter Collignon; The Canberra Hospital. New South Wales: Richard Benn (Royal Prince Alfred Hospital); Ian Chambers (Douglas Hanly Moir Pathology); Sharon Chen (Westmead Hospital); Nancy Deller (Wollongong Hospital); Deo DeWitt (Gosford Hospital); John Ferguson (John Hunter Hospital); Iain Gospell (Liverpool Hospital); Thomas Gottlieb (Concord Hospital); Catriona Halliday (Westmead Hospital); Juliette Holland (Mayne Laverty Pathology); Alison Kessons (New Children’s Hospital, Westmead); Richard Lawrence (St. George Hospital); Deborah Marriot (St. Vincent’s Hospital, Sydney); Wieland Meyer (Westmead Hospital); Peter Newton (Wollongong Hospital); Quoc Nguyen (St. Vincent’s Hospital, Sydney); Pamela Palasanthrian (Sydney Children’s Hospital); Robert Pickles (Infectious Diseases Physician, Taree); Robert Pritchard (Royal North Shore Hospital); Tania Sorrell (Westmead Hospital); Lex Tierney (John Hunter Hospital); Voula Tomasotos (Liverpool Hospital); Robert Vaz (Orange Base Hospital); Kerry Weeks (Royal North Shore Hospital). Queensland: Anthony Allworth (Royal Brisbane Hospital); Christopher C出局er (The Prince Charles Hospital); Joan Faouagl (Royal Brisbane Hospital); Barbara Johnson (Princess Alexandra Hospital); David Looke (Princess Alexandra Hospital); Joseph McCormack (The Mater Adult Hospital); Graeme Nimmo (Princess Alexandra Hospital); Gabrielle O’Kane (The Prince Charles Hospital); E. Geoffrey Playford (Princess Alexandra Hospital); Jennifer Robson (Sullivan and Nicholson’s Hospital). South Australia: David Ellis (Women’s and Children’s Hospital); Rosemary Handke (Women’s and Children’s Hospital); Karen Rowlands (Royal Adelaide Hospital); David Shaw (Royal Adelaide Hospital); Tasmania: Louise Cooley (Royal Hobart Hospital); Erica Cox (Launceston General Hospital); Alistair McGregor (Royal Hobart Hospital); Victoria: Claire Franklin (Alfred Hospital); Cathy Joseph (St. Vincent’s Hospital, Melbourne); Tony Korman (Monash Medical Centre); Orla Morrissey (Alfred Hospital); Monica Slavin (Peter MacCallum Cancer Centre); Denis Speelman (Alfred Hospital); Bryan Speed (Austin and Repatriation Hospitals); Harsha Sheerey (St. Vincent’s Hospital, Melbourne). Western Australia: Peter Boan (Royal Perth Hospital); John Dyer (Fremantle Hospital); Christopher Heath (Royal Perth Hospital); Dianne Gardam (Royal Perth Hospital); Duncan McLennan (Fremantle Hospital); Roman Murray (Royal Perth Hospital); Todd Pryce (Royal Perth Hospital).
S. apiospermum infections occur worldwide, ranging from localized mycetomas to deep-seated disease such as cerebral abscesses (6,7). This species also colonizes the respiratory tract of 10% of patients with cystic fibrosis and chronic suppurative lung disease (8–10). On the basis of genetic data, a new species, S. aurantiacum, was proposed for a subset of isolates previously identified as S. apiospermum (11). S. prolificans infections are geographically more restricted than those caused by S. apiospermum, being most prevalent in Australia, Spain, and the United States (12–15). S. prolificans typically causes localized infections in immunocompetent hosts but rapidly fatal disseminated infections in the immunocompromised among whom it has been associated with nosocomial outbreaks (3,12–17).

Since scedosporiosis, in particular that caused by S. prolificans, is often refractory to treatment (3,5), preventive strategies are of paramount importance. However, the epidemiology and mode of transmission of infection are not well understood. Furthermore, the environmental reservoir of S. prolificans is unknown. Molecular typing techniques now provide the means to elucidate the epidemiology of Scedosporium infections and to investigate potential case clusters (16,18,19). Strains recovered from patients with cystic fibrosis have demonstrated a high degree of genetic variability (10,20), although a single genetic profile predominated in 1 study (8). The degree of genetic variation within S. prolificans is more controversial. Two studies have reported low to no intraspecies genetic heterogeneity (16,21), while a third noted substantial genetic diversity (19). The results of these studies may be biased because they included only small numbers of isolates from specific patient populations. Genetic variability among S. aurantiacum has not yet been studied.

In this study, we used 4 molecular tools to examine genetic variation among a large number of Australian clinical Scedosporium isolates: 1) internal transcribed spacer (ITS)–based restriction fragment length polymorphism (ITS-RFLP) analysis; 2) DNA sequence analysis of the ITS region (selected isolates); 3) PCR fingerprinting using the microsatellite specific core sequence of phage M13; and 4) amplified fragment length polymorphism (AFLP) analysis (isolates from suspected case clusters). We also searched for the newly described species, S. aurantiacum and for genetic clustering of strains according to their geographic origin, body site from which they were cultured, and ability to cause invasive disease.

Materials and Methods

Scedosporium Isolates and Data Collection

A total of 146 Scedosporium isolates were studied (online Technical Appendix, available from www.cdc.gov/EID/content/14/2/282-Techapp.pdf). Forty-six were from the culture collection at the Clinical Mycology Laboratory, Centre for Infectious Diseases and Microbiology Laboratory Services, Westmead Hospital, Sydney, Australia. For these isolates, the following data were captured: demographic information, patient coexisting conditions and risk factors (summarized in the online Technical Appendix). The remaining 100 isolates were obtained through a national, prospective, laboratory-based surveillance for scedosporiosis in Australia (the Australian Scedosporium [AUSCEDO] Study) from January 2003 to December 2005. The following data were collected: clinical status, risk factor (defined according to published risk factors for scedosporiosis [4,12–15]), major comorbidity (based on the International Classification of Diseases, 10th revision, Australian Modification [ICD-10 AM] diagnostic classification system [22]), isolated species, treatment and outcome. Scedosporium strains obtained from a single colony from the primary isolation plate from all patients were forwarded to the Molecular Mycology Research Laboratory, Westmead Hospital, for genotyping. Isolates were identified as S. prolificans or S. apiospermum by standard phenotypic methods (23). Species were confirmed as S. prolificans or S. apiospermum, and S. aurantiacum was identified (11) by ITS-RFLP analysis.

Definitions

An episode of scedosporiosis was defined as the incident isolation of Scedosporium spp. from any body site. Two or more episodes, fulfilling the case definition and occurring in different patients that were epidemiologically linked were defined as a potential case cluster. Invasive disease was defined according to the European Organization for Treatment of Cancer/Mycoses Study Group criteria for “definite” or “probable” infection (24). All other patients not fulfilling these criteria, including those with “possible” infection were considered colonized. Coincident hospital renovations or construction was considered to be a potential risk factor if major work was undertaken within 3 months before the isolation of Scedosporium spp. from a patient.

Description of 2 Potential Case Clusters

The first potential case cluster involved 8 patients located in the same hematology/hemopoietic stem cell transplant (HSCT) unit at the Alfred Hospital, a large university hospital in Melbourne (September 2000–October 2001; [15]). The second consisted of 3 patients located in the same hematology/HSCT ward at Westmead Hospital a major university hospital in Sydney (September 2003–January 2004; unpub. data). Details of the patients involved in these suspected case clusters are summarized in the online Technical Appendix). On each occasion, patient isolates were submitted for genetic analyses to inform infection control responses (see Results).
Genomic DNA Extraction and ITS-RFLP Analysis

Genomic DNA was isolated as described previously (18). The ITS1, 5.8S, and ITS2 regions of the rDNA gene cluster were amplified with the primers SR6R and LR1 (Table 1) as described previously (25). Amplicons were double digested with the restriction endonucleases Sau96I and HhaI (New England BioLabs, Ipswich, MA, USA) in accordance with the manufacturer’s recommendations. Digested products were separated by electrophoresis in 3% agarose gels at 100 V for 3–4 h. Banding patterns were analyzed visually.

ITS Sequencing

Eleven isolates, representative of each of 3 ITS-RFLP patterns obtained, were selected for ITS sequencing: ITS-RFLP profile A (S. prolificans, WM 06.378, WM 06.440, and WM 06.393), ITS-RFLP profile B (S. apiospermum, WM 06.389, WM 06.471, and WM 06.497), and ITS-RFLP profile C (S. aurantiacum, WM 06.388, WM 06.482, WM 06.495, WM 06.496, and WM 06.498). The ITS region was amplified as described above and commercially sequenced in both directions by using SR6R or LR1 (Table 1) as forward and reverse primers.

PCR Fingerprinting

The minisatellite-specific core sequence of the wild-type phage M13 was used as a single primer for PCR fingerprinting (Table 1). Amplification reactions were performed as previously described (18). Blank control tubes containing all reagents except template DNA were included for each run; each sample was analyzed at least twice. PCR products were separated by electrophoresis on 1.4% agarose gels at 60 V for 14 cm. Strains were defined to be identical if their PCR fingerprinting profiles had a similarity of ≥97% ( = 1 band difference). Reproducibility of the PCR fingerprinting technique was accessed by re-amplifying 1 strain of each of the 3 Scedosporium spp. with all PCR amplifications carried out and re-running those on each gel.

AFLP Analysis

AFLP analysis was performed as described previously by using either EcoRI-GT 6-FAM-labeled and Msel-GT or EcoRI-TC 6-FAM-labeled and Msel-CA as selective primer pairs (QIAGEN, Valencia, CA, USA; Table 1) (26). All samples were analyzed by using the ABI Prism 3730 system (Applied Biosystems, Foster City, CA, USA). Data collation, fragment sizing, and pattern analyses were performed with GeneMapper software version 3.5 (Applied Biosystems). Only electrophoregram peaks above 1,000 fluorescent units were scored for the presence or absence of bands of the same size (range 50–500 bp) relative to the GeneScan 500 LIZ DNA size standard (Applied Biosystems). Only bands detected in duplicate AFLP experiments were included in the analysis.

Data Analysis

Clinical Data

Statistical analysis was performed by using SPSS version 10.0.0.7 (SPSS, Chicago, IL, USA) and EpiInfo version 6.0 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Proportions were compared by using the x² or Fisher exact test. A p value <0.05 was statistically significant.

ITS Sequences

ITS sequences obtained from 11 isolates (see above) were aligned with the ITS sequences of the following reference strains obtained from GenBank: S. apiospermum CBS 101.22 (accession no. AJ888435), S. aurantiacum FMR 8630 (accession no. AJ888440), S. aurantiacum IHEM 15458 (accession no. AJ888441) and S. prolificans CBS 114.90 (accession no. AY882369) as well as 2 outgroup sequences: Pseudallescheria africana CBS 311.72 (accession no. AJ888425), and Petriella setifera CBS 164.74 (accession no. AY882352). Phylogenetic analyses were performed by using PAUP* version 4.06.10 (27).

PCR Fingerprinting Patterns and AFLP Fragments

PCR fingerprinting patterns were analyzed by using the 1D gel analysis module (BioGalaxy [BioAware, Han- nut, Belgium]) in BioloMICS version 7.5.30 (BioAware). Images were normalized for lane to-lane differences in mobility by the alignment of patterns obtained on multiple loadings of the 1kb DNA size marker (GIBCO-BRL, Gaithersburg, MD, USA). The unweighted-pair group method by using arithmetic averages and the procedures of Nei and Li (28), both implemented in BioloMICS, were used to generate dendograms based on the coefficient of similarity (29) between the isolates. In addition, principal coordinate analysis (PcoA; BioloMICS) was conducted to give an overall representation of the observed strain variation. AFLP fragments were analyzed with BioloMICS.

Results

A total of 146 Scedosporium isolates from 120 episodes (119 patients) were studied (online Technical Appendix). Demographic data were available for 108 (90%) episodes and coexisting conditions and risk factor data for 115 (95.8%). Most episodes were reported from New South Wales (64.2%), followed by Victoria (19.2%) and Western Australia (9.2%). The male: female ratio was 1.3: 1. The major patient coexisting conditions and known risk factors for scedosporiosis are summarized in the online Technical Appendix. Thirty-nine patients (32.7%) had no underlying medical condition. Coincident building construction was noted in 27 cases (22.5%). Scedosporium isolates were associated with invasive disease in 46 (38.3%) instances;
the remaining 74 (61.7%) were isolated from patients who were colonized (Table 2).

Molecular Typing of Scedosporium isolates

All 146 isolates were examined by ITS-RFLP analysis and PCR fingerprinting. ITS sequencing was performed on 11 strains as described above. AFLP analysis was performed only for selected S. prolificans isolates, including the isolates of the suspected case clusters and isolates representative of the S. prolificans branches identified by PCR fingerprinting (online Appendix Figure 1, available from www.cdc.gov/EID/content/14/2/282-appG1.htm).

<table>
<thead>
<tr>
<th>Table 1. Primer and adaptor oligonucleotide sequences used in the study</th>
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<tbody>
<tr>
<td>Primer or adaptor oligonucleotide</td>
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<tr>
<td>-----------------------------------</td>
</tr>
<tr>
<td>rDNA primers SR6R</td>
</tr>
<tr>
<td>MseI-RFLP primer LR1</td>
</tr>
<tr>
<td>M13 fingerprinting primer Phage M13</td>
</tr>
<tr>
<td>EcoRI adapters EA1</td>
</tr>
<tr>
<td>MseI adapters MA1</td>
</tr>
<tr>
<td>Preselective primers EcoRI-T</td>
</tr>
<tr>
<td>MseI-C</td>
</tr>
<tr>
<td>Selective primers EcoRI-TG</td>
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<tr>
<td>MseI-G</td>
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<tr>
<td>Selective primers EcoRI-GT</td>
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</table>

ITS Sequencing

Sequencing of the ITS 1, 5.8S, and ITS2 regions of the 11 strains, representative of each of the 3 ITS-RFLP profiles found the following results: BLAST searches against the corresponding GenBank reference sequences identified strains: WM 06.389 (accession no. EF639870), WM 06.497 (accession no. EF639872), and WM 06.471 (accession no. EF639871) (ITS-RFLP profile B) as S. apiosper-
associated with isolation of *S. proliﬁcans* compared with non-*proliﬁcans* *Scedosporium* spp. (OR 11.5, 95% CI 2.4, 74.5; p<0.001; data not shown).

**Molecular Epidemiology**

**Strain Typing**

PCR ﬁngerprinting delineated 3 major clusters concordant with *S. apiospermum*, *S. aurantiacum*, and *S. proliﬁcans* (online Appendix Figure 1; Figure 1, panel B; Figure 3). Clusters corresponding to *S. aurantiacum* and *S. proliﬁcans* were substantially more densely grouped than the *S. apiospermum* cluster (Figure 3). PCR ﬁngerprinting proﬁles showed polymorphisms within each of the 3 species, allowing for a clear differentiation, by using a “cut-off point” of ≥97% similarity. Multiple isolates from the same patient obtained from diﬀerent anatomic sites (online Technical Appendix) had identical or ≥97% similarity between their PCR ﬁngerprints, except for 1 patient (patient 118). In 8 instances, PCR ﬁngerprinting showed that patients were infected with 2 diﬀerent strains: (patients 1, 10, 27, 57, 83, 99, 118 [online Appendix Figure 1, online Technical Appendix]). For all species, genetic proﬁles were independent of geographic origin, body site of isolation or whether the patient was infected or colonized (online Appendix Figure 1). Proﬁles were also independent of patient comorbidity and risk factors for scedosporiosis (data not shown). Intraspecies PCR ﬁngerprinting variation was highest for *S. apiospermum* (58%) followed by *S. proliﬁcans* (45%) and *S. aurantiacum* (28%) (online Appendix Figure 1).

**Examination of Isolates from Suspected Case Clusters**

Twelve isolates from 2 presumptive case clusters of *S. proliﬁcans* infection (Alfred Hospital, Melbourne pa...
Epidemiologic investigation of *Scedosporium* infection requires accurate identification and typing. *S. apiospermum*, *S. aurantiacum*, and *S. prolificans* were clearly distinguished from each other by PCR fingerprinting and ITS-RFLP analysis. This is consistent with previous rDNA sequence-based studies (30,33,34). The observation of 2 distinct genetic groups, corresponding to *S. aurantiacum* and *S. apiospermum*, supports the proposal that *S. aurantiacum* be designated a separate species (11). This proposal is also supported by the 5%–10% ITS sequence variation found between *S. aurantiacum* and *S. apiospermum* compared to an absence of intraspecies variation in *S. aurantiacum* and *S. prolificans* and a 2.2% variation in *S. apiospermum* (30,32; current study).

Using PCR fingerprinting, intraspecies variation was greatest (58%) among *S. apiospermum* isolates (Figure 3). This diversity is generally consistent with the high degree of polymorphism (15–20 genotypes) previously found (10,20,32). In contrast, genetic variation was lowest (28%) among the *S. aurantiacum* isolates (online Appendix Figure 1; Figure 3). Nevertheless, PCR fingerprinting polymorphisms clearly differentiated all 30 strains (online Appendix Figure 1). Further genotyping studies of a greater number of and more geographically diverse *S. aurantiacum* isolates are warranted.

The intraspecies PCR fingerprint variation in *S. prolificans* (45%) was greater than that in *S. aurantiacum* but less than that in *S. apiospermum*. Given that *S. aurantiacum* is phylogenetically more closely related to *S. apiospermum* than to *S. prolificans* (11,33; current study), this result was unexpected. It may be due to different evolutionary pressures acting on the 3 different species or the relatively small numbers of *S. aurantiacum* isolates studied to date. The moderate genetic diversity among *S. prolificans* confirms previous findings (19). Despite the observed

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**Table 2. Selected characteristics for 120 isolations (episodes) of *Scedosporium* spp.**

<table>
<thead>
<tr>
<th>Characteristic*</th>
<th>No. <em>Scedosporium prolificans</em>; n = 75 (%)†</th>
<th>No. <em>S. apiospermum</em>; n = 29 (%)†</th>
<th>No. <em>S. aurantiacum</em>; n = 23 (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>40 (53.3)</td>
<td>12 (48)</td>
<td>8 (34.8)</td>
</tr>
<tr>
<td>Risk factor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgery ≤30 d</td>
<td>3 (4)</td>
<td>1 (4)</td>
<td>–</td>
</tr>
<tr>
<td>Trauma</td>
<td>5 (6.7)</td>
<td>–</td>
<td>1 (4.4)</td>
</tr>
<tr>
<td>Clinical status‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasive disease</td>
<td>39 (52)</td>
<td>4 (16)</td>
<td>4 (17.4)</td>
</tr>
<tr>
<td>Colonization</td>
<td>36 (48)</td>
<td>21 (84)</td>
<td>19 (82.6)</td>
</tr>
<tr>
<td>Body site of isolation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>18 (24)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Eye</td>
<td>2 (2.7)</td>
<td>–</td>
<td>1 (4.4)</td>
</tr>
<tr>
<td>Skin/soft tissue</td>
<td>4 (5.3)</td>
<td>1 (4)</td>
<td>2 (8.7)</td>
</tr>
<tr>
<td>Lung/respiratory tract</td>
<td>15 (20)</td>
<td>10 (40)</td>
<td>12 (52.2)</td>
</tr>
<tr>
<td>Ear</td>
<td>4 (5.3)</td>
<td>10</td>
<td>5 (21.2)</td>
</tr>
</tbody>
</table>

*Some patients had *Scedosporium* isolated from more than 1 body site.
†Refers to no. episodes in which each species was isolated. The total no. of isolates was 146 comprising 83 *S. prolificans*, 33 *S. apiospermum*, and 30 *S. aurantiacum*.
‡More than 1 *Scedosporium* spp. was isolated from 4 patients.
polymorphisms, PcoA of PCR fingerprinting profiles showed dense clustering for *S. prolificans* (Figure 3), which is consistent with the low to absent intraspecies variability in *S. prolificans* found by others (20,30). These apparently contradictory findings emphasize the importance of choosing the optimum molecular typing tool with the most appropriate discriminatory power for the organism or species being studied.

The high degree of intraspecies variation detected by PCR fingerprinting and AFLP analysis supports the use of these methods to establish genetic relatedness between isolates recovered from different patients or multiple isolates from the same patient. In comparison, the variation detected by ITS-RFLP analysis and ITS sequencing corresponded to interspecies variation, which makes those techniques ideal for identification of any given isolate to the species level. Individual patients are most likely infected or colonized with genetically distinct strains (19–21; this study). However, 8 patients were infected or colonized by at least 2 strains as reflected by their different genetic profiles (online Technical Appendix). Possible explanations include concomitant infection by multiple strains from which only a restricted number were recovered, or colonization by 1 strain followed by infection or colonization with a second strain of a different genotype. Longitudinal genotyping studies are required to determine the likelihood that persistence of $\geq 1$ genotypes later leads to clinically important infection or whether the disease is more likely to be caused by an unrelated genotype. In this context, the development of a multilocus sequence typing scheme for *Scedosporium*, as has been developed for *Candida* spp. (35), would be of great advantage to overcome interlaboratory reproducibility problems, which are known to be associated with PCR fingerprinting or AFLP data. However, developing such a scheme remains cumbersome due to the current lack of genomic data of *Scedosporium* spp.

For all 3 *Scedosporium* spp., there was no clustering of strains according to their geographic or body site of origin or by their ability to cause invasive disease, which is in agreement with previous findings for *S. apiospermum* (20,30) and *S. prolificans* (16,17,21). Of note, no specific genotypes were associated with underlying medical conditions or risk factors. Compared with *S. apiospermum* and *S. aurantiacum* *S. prolificans* was more frequently associated with coincident hospital renovation, and invasive disease, had a greater predilection to cause disseminated infection and was the predominant species isolated from blood and other sterile sites (12–16,36; current study). Our preliminary observations indicate that the epidemiology and clinical relevance of recovering *S. aurantiacum* may be similar to that of *S. apiospermum*. *S. aurantiacum* has been reported to colonize the respiratory tract of at-risk patients (8).
In addition to PCR fingerprinting, we applied AFLP analysis to investigate the possibility of 2 case clusters caused by \textit{S. prolificans}. AFLP analysis was chosen as an independent technique using 2 combinations of selective primers (Table 1), which have been previously shown to have good discriminatory power for fungal strain differentiation (26). Both techniques, previously used to identify outbreak strain clusters in the recent cryptococcosis outbreak on Vancouver Island (37), generated in the current situation distinct patterns from all \textit{S. prolificans} isolates except serial isolates obtained from the same patient (online Appendix Figures 1, 2). These findings exclude the occurrence of nosocomial outbreaks or any close relationship with the nonoutbreak isolates, a result similar to those obtained previously (38). Overall nosocomial acquisition of infection has been demonstrated in only 2 instances (16, 17). \textit{Scedosporium} spp. have rarely been isolated from hospital air or from indoor or outdoor surface samples (13, 39, 40, current study), which raises questions about the mode of acquisition by patients and the mechanisms of the selection of this specific fungus as an infectious agent from among the high biodiversity of environmental molds.

In conclusion, ITS-RFLP analysis is a powerful tool for distinguishing between isolates of the new species \textit{S. aurantiacum} and \textit{S. apiospermum}. PCR fingerprinting and AFLP analysis are useful techniques for determining genetic relatedness between \textit{Scedosporium} isolates and for investigating potential case clusters.

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Dr Delhaes is the head of the filamentous fungal research group EA3609 at the Pasteur Institute in Lille, France. Her major research interests are systemic mycoses due to \textit{Aspergillus} and \textit{Scedosporium}, including molecular epidemiology, nosocomial aspects of the diseases, and oxidative stress response.

References


Address for correspondence: Wieland Meyer, Molecular Mycology Research Laboratory, Westmead Hospital, Centre for Infectious Diseases and Microbiology, Institute of Clinical Pathology and Medical Research, Level 3, Room 3114A, Darcy Rd, Westmead, New South Wales 2145, Australia; email: w.meyer@usyd.edu.au

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