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Original Article

Semi-automated repetitive sequence-based PCR amplification for species of the *Scedosporium apiospermum* complex

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Abstract

Purpose: The *Scedosporium apiospermum* species complex usually ranks second among the filamentous fungi colonizing the airways of patients with cystic fibrosis (CF), but little is known about the molecular epidemiology of the airway colonization.
Methods: Polymerase chain reaction (PCR) amplification of repetitive sequences (rep-PCR) was applied to the retrospective analysis of a panel of isolates already studied by random amplification of polymorphic DNA (RAPD) and comprising 63 isolates recovered from sputa from 9 CF patients. Results were compared to those obtained previously by RAPD, and herein by beta-tubulin (TUB) gene sequencing and Multilocus Sequence Typing (MLST).
Results: Within the panel of isolates studied, *S. apiospermum sensu stricto* and *Scedosporium boydii,* as expected, were the predominant species with 21 and 36 isolates, respectively. Four isolates from one patient were identified as *Scedosporium aurantiacum,* whereas two isolates belonged to the *Pseudallescheria ellipsoidea* subgroup of *S. boydii*. rep-PCR analysis of these isolates clearly differentiated the three species and *P. ellipsoidea* isolates, whatever the rep-PCR kit used, and also permitted strain differentiation. When using the mold primer kit, results from rep-PCR were in close agreement with those obtained by MLST. For both *S. apiospermum* and *S. boydii,* 8 genotypes were differentiated by rep-PCR and MLST compared to 10 by RAPD. All *S. aurantiacum* isolates

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shared the same RAPD genotype and exhibited the same rep-PCR profile and sequence type.
Conclusions: These results illustrate the efficacy of rep-PCR for both species identification within the *S. apiospermum* complex and genotyping for the two major species of this complex.
Abstract presentation: Part of this work was presented during the 18th Congress of the International Society for Human and Animal Mycology, Berlin (Germany), June 2012.
S. Giraud, C. Godon, A. Rougeron, J.P. Bouchara and L. Favennec are members of the *ECMM/ISHAM working group on Fungal respiratory infections in Cystic Fibrosis* (*Fri-CF*).

Key words: *Scedosporium apiospermum* species complex, Cystic fibrosis, Species identification, Genotyping, rep-PCR.

Introduction

Prognosis in cystic fibrosis (CF), the major inherited disease in the Caucasian population, mainly depends on the severity of the lesions of the lungs. Because of the thickening of the bronchial mucus and of the impairment of the mucociliary clearance, the respiratory tract of CF patients is often colonized by various microorganisms, sometimes leading to respiratory infections which are the major cause of morbidity and mortality in this context.[1] Considerable attention has been paid during the past decades to the prevention and treatment of bacterial respiratory infections, and progress in this field resulted in a marked increase in life expectancy.[2] Nevertheless, continued improvement in life expectancy requires a better knowledge of the causative agents of these infections and greater attention should be paid to nonbacterial pathogens, especially fungi.[3]

With a frequency ranging from 4.5% to 11.6%, *Scedosporium apiospermum*, which was initially considered the anamorph of *Pseudallescheria boydii,* usually ranks second among the filamentous fungi colonizing the CF airways, after *Aspergillus fumigatus*.[4–7] In CF, this fungal colonization may result in endobronchitis and allergic bronchopulmonary mycoses.[4,8] Moreover, it may compromise the success of lung transplantation due to the capacity of the fungus to cause severe and often fatal disseminated infections in immunocompromised individuals[9–11] and to the limited efficacy of current antifungals.[12–14]

Limited information is presently available regarding the natural history of the airway colonization by *S. apiospermum* in CF. Recent taxonomic studies revealed that *S. apiospermum* and *P. boydii* are two distinct species, and three additional species were identified.[15–17] Therefore, *S. apiospermum* is now considered a species complex comprising five distinct species, namely *Scedosporium apiospermum sensu stricto, Scedosporium aurantiacum, Scedosporium dehoogii, P. boydii* (including the *Pseudallescheria ellipsoidea* subgroup), and *Pseudallescheria minutispora*, which are now called *Scedosporium boydii* and *Scedosporium minutisporum*.[18] Regarding the molecular epidemiology of airway colonization / respiratory infections in CF, it was shown that apart from *S. dehoogii* which has never been reported in respiratory diseases, all these fungi are usually responsible for a chronic colonization of the airways.[19,20] However, these results were obtained by random amplification of polymorphic DNA (RAPD) which is considered a poorly reproducible method. A multi-locus sequence typing (MLST) scheme has been proposed for genotype studies, based on the sequencing of five distinct loci.[21] Recently, the semi-automated DiversiLab system based on polymerase chain reaction (PCR) amplification of repetitive sequences (rep-PCR) followed by separation of the amplified products by capillary electrophoresis has been successfully applied to species identification in various fungal groups including *Candida* yeasts, *Aspergillus* and *Fusarium* species,[22–24] as well as to strain differentiation within *Candida* and *Aspergillus* species.[23,25] Here this method was applied to the retrospective analysis of multiple or sequential isolates belonging to the *S. apiospermum* species complex recovered from sputum samples collected from CF patients, and previously analyzed by RAPD, and results were compared to those obtained by beta-tubulin gene sequencing and MLST.

Materials and methods

Microorganisms and culture conditions

This study was performed using a panel of 63 multiple (from the same clinical sample) or sequential (from successive samples from the same patient) isolates recovered from sputum samples collected from nine CF patients, and previously studied by RAPD[20] (Table 1). Among this set of isolates, RAPD differentiated 12 genotypes; a unique genotype was found for seven patients, while one patient (Patient P1) exhibited a largely dominant genotype

Table 1. Isolates studied and their rep-PCR genotypes compared to previously determined RAPD genotypes and to herein identified MLST sequence types.

Patient number[1]	Sampling date (yyyy/mm/dd)	IHEM number	Species identification	Genbank accession number[2]	rep-PCR genotype[3]		MLST ST	RAPD genotype[1]
					Mold kit	*Aspergillus* kit		
P1	1998/01/09	14263	*S. boydii*	*AJ889990*	3	5	10	1
		14264	*S. boydii*	KC812533	3	5	10	1
		14266	*S. boydii*	KC812534	3	5	10	1
		14267	*S. boydii*	KC812535	3	5	10	1
	1998/02/16	14369	*S. boydii*	KC812536	3	5	10	1
	1998/03/10	14457	*S. boydii*	KC812537	3	5	10	1
	1998/06/10	14638	*S. boydii*	*AJ889998*	3	5	10	2
	1998/07/31	14756	*S. boydii*	KC812538	3	5	10	1
		14758	*S. boydii*	*AJ890000*	3	5	10	3
		14759	*S. boydii*	KC812539	3	5	10	1
	1998/09/04	15142	*S. boydii*	KC812540	3	5	10	1
	1999/01/13	15582	*S. boydii*	KC812541	3	5	10	1
		15583	*S. boydii*	KC812542	3	5	10	1
P2	1999/01/13	49900353/1[4]	*P. ellipsoidea*	KC812543	7	8	/	4
		49900353/2[4]	*P. ellipsoidea*	KC812544	7	8	/	4
P3	1998/02/24	14452	*S. boydii*	KC812545	4	5	3	5
		14453	*S. boydii*	KC812546	4	5	3	5
		14455	*S. boydii*	KC812547	4	5	3	5
	1998/05/26	14624	*S. boydii*	KC812548	4	5	3	5
		14626	*S. boydii*	KC812549	4	5	3	5
		14627	*S. boydii*	KC812550	4	5	3	5
	1998/06/06	14628	*S. boydii*	KC812551	4	5	3	5
		14629	*S. boydii*	KC812552	4	5	3	5
		14631	*S. boydii*	KC812553	4	5	3	5
		14632	*S. boydii*	KC812554	4	5	3	5
	1998/06/15	14633	*S. boydii*	KC812555	4	5	3	5
		14634	*S. boydii*	KC812556	4	5	3	5
		14635	*S. boydii*	KC812557	4	5	3	5
		14636	*S. boydii*	KC812558	4	5	3	5
		14637	*S. boydii*	KC812559	4	5	3	5
	1998/11/09	15464	*S. boydii*	KC812560	4	5	3	5
P4	1998/01/08	14268	*S. apiospermum*	*AJ889991*	8	3	37	6
		14269	*S. apiospermum*	KC812561	8	3	37	6
		14270	*S. apiospermum*	KC812562	8	3	37	6
	1998/01/21	14273	*S. apiospermum*	KC812563	8	3	37	6
		14275	*S. apiospermum*	KC812564	8	3	37	6
		14276	*S. apiospermum*	KC812565	8	3	37	6
	1999/02/15	15643	*S. apiospermum*	KC812566	8	3	37	6
P5	1998/09/22	15149	*S. apiospermum*	*AJ890002*	9	4	39	8
		15151	*S. apiospermum*	KC812567	9	4	39	8
P6	1998/10/26	15458	*S. aurantiacum*	*AJ890135*	2	6	47	9
		15459	*S. aurantiacum*	KC812568	2	6	47	9
		15460	*S. aurantiacum*	KC812569	2	6	47	9
		15461	*S. aurantiacum*	KC812570	2	6	47	9
P7	1998/01/19	14357	*S. boydii*	KC812571	6	5	11	10
	1998/09/22	15545	*S. boydii*	KC812572	6	5	11	10
P8	1998/03/04	14462	*S. apiospermum*	*AJ889995*	10	1	38	11
		14463	*S. apiospermum*	KC812573	10	2	38	11
		14465	*S. apiospermum*	KC812574	10	1	38	11
	1998/08/11	14762	*S. apiospermum*	KC812575	11	4	36	12

Table 1. – *continued*

Patient number[1]	Sampling date (yyyy/mm/dd)	IHEM number	Species identification	Genbank accession number[2]	rep-PCR genotype[3] Mold kit	rep-PCR genotype[3] *Aspergillus* kit	MLST ST	RAPD genotype[1]
		14763	*S. apiospermum*	KC812576	11	4	36	12
		14764	*S. apiospermum*	KC812577	11	4	36	12
	1998/09/22	15146	*S. apiospermum*	KC812578	11	4	36	12
		15148	*S. apiospermum*	KC812579	11	4	36	12
	1998/11/10	15551	*S. apiospermum*	KC812580	11	4	36	12
		15552	*S. apiospermum*	KC812581	11	4	36	12
		15553	*S. apiospermum*	KC812582	10	4	38	11
	1998/12/03	15555	*S. apiospermum*	KC812583	11	4	36	12
P9	1998/02/16	14358	*S. boydii*	*AJ889993*	*5*	*5*	12	15
		14359	*S. boydii*	KC812584	*5*	*5*	12	15
		14360	*S. boydii*	KC812585	*5*	*5*	12	15
		14361	*S. boydii*	JQ691025	*5*	*5*	12	15
		14362	*S. boydii*	KC812586	*5*	*5*	12	15
Environment[5]	1991	21148	*S. minutisporum*	*AJ890122*	1	7	/	/

[1]Patient numbers and RAPD genotypes as previously published.[20]

[2]Accession numbers correspond to the nucleotide sequence of TUB region of the beta-tubulin gene which was sequenced for all isolates in this work, except italicized numbers which correspond to TUB sequences deposited in Genbank by Gilgado et al.[15]

[3]rep-PCR genotypes were numbered according to their order in the corresponding phylogenetic trees (see Fig. 3).

[4]Isolates not deposited at the IHEM culture collection and designated by Angers University Hospital (Angers, France) identification number.

[5]Type strain of *S. minutisporum* (also called FMR 4072) isolated in 1991 from sediment from Tordera river (Barcelona, Spain) and identified as *S. minutisporum* by Gilgado et al.[15]

associated with two others, each found only once, and two distinct genotypes were detected successively, but once associated, for the last patient (Patient P8). No genotypes were shared by the patients. These isolates preserved by freeze-drying in our laboratory and deposited at the Institute of Hygiene and Epidemiology-Mycology section (IHEM) culture collection (Scientific Institute of Public Health, Brussels, Belgium), were initially identified on a morphological basis as belonging to the *S. apiospermum* complex. Additionally, the type strain of *S. minutisporum* 21148, isolated from sediments from the Tordera river (Barcelona, Spain)[15] and obtained from the IHEM culture collection was included in our set of isolates.

Isolates were cultivated on yeast extract-peptone-dextrose (YPD) agar (containing in g/l: yeast extract, 5; peptone, 10; glucose, 20; and agar, 20) plates supplemented with 0.5 g/l chloramphenicol. After incubation (7 days, 37°C), the mycelium was scraped and inoculated in YPD broth. Cultures were incubated for 10 days at 37°C. Finally the fungal mat was harvested and ground with a mortar and pestle in liquid nitrogen.

Repetitive sequence-based PCR

Fungal DNA was extracted from ground mycelium using the MO-BIO Ultraclean preparation kit (Ozyme, Saint-Quentin en Yvelines, France). DNA was quantified at 260 nm using a Nanodrop spectrophotometer (NanoDrop technologies, Wilmington, DE). Repetitive DNA sequences were amplified by PCR using two primer sets from bioMérieux (Craponne, France): the Mold DNA fingerprinting primer kit proposed for the analysis of all mold species, and the *Aspergillus*-specific DNA fingerprinting primer kit which uses primers specifically designed for *A. fumigatus*. Amplicons were separated by capillary electrophoresis and electrophoretic profiles were compared using the DiversiLab Healthcare software version 3.41 (bioMérieux). Phylogenetic trees were established using the unweighted pair group method with arithmetic mean (UPGMA). The interpretative criteria provided by the manufacturer led us to categorize the isolates as indistinguishable, closely related or different (DiversiLab user's guide). Isolates with electrophoretic profiles differing by at least three peaks were categorized as different. Isolates were categorized as indistinguishable and belonging to the same genotype when they shared the same electrophoretic profile, and isolates with electrophoretic profiles differing by one or two bands were categorized as closely related. Rep-PCR genotypes were numbered according to their order in the phylogenetic trees.

Species identification within the *S. apiospermum* complex

Molecular identification was performed as described by Zouhair et al.[19] by PCR amplification of part of

the beta-tubulin gene (TUB). Amplified products were purified using the NucleoSpin extract II kit (Macherey-Nagel, Hoerdt, France) and commercially sequenced in both directions. The obtained sequences were analyzed by individual BLASTn (Basic Local Alignment Search Tool) searches using the NCBI (National Center for Biotechnology Information) BLAST database. Ninety-nine to 100% sequence similarities to isolates FMR 8630, FMR 4072, FMR 8535, FMR 8537, and FMR 7884 analyzed by Gilgado et al.[15] were used to identify the present isolates as *S. aurantiacum*, *S. minutisporum*, *S. apiospermum*, *S. boydii,* and *P. ellipsoidea,* respectively. All sequences were deposited in the Genbank database under the accession numbers indicated in Table 1.

Multilocus Sequence Typing (MLST)

All *S. boydii* not including *P. ellipsoidea* (n = 36), *S. apiospermum* (n = 21) and *S. aurantiacum* isolates (n = 4) were analyzed by MLST as previously described by Bernhardt et al.[21] or according to recommendations of the Fungal MLST Database (http://mlst.mycologylab.org/) for *S. aurantiacum* isolates. For all the isolates, DNA was amplified by PCR using five primer pairs targeting part of the genes encoding actin (*ACT*), calmodulin (*CAL*, exon 3–4), the second largest subunit of RNA polymerase II gene (*RPB2*), ß-tubulin (*BT2*, exon 2–4) and the manganese superoxide dismutase (*SOD2*). Moreover, for *S. aurantiacum* isolates, an additional sequence in the elongation factor 1 alpha *(EF1α)* gene was amplified. In addition, to optimize the amplification of *RPB2* and *BT2* loci for *S. apiospermum* isolates, two new primer pairs were designed from the whole genome sequence of *S. apiospermum* strain IHEM 14462[26]: RPB2–5F 5′-GACGACCGTGATCACTTTGG-3′ and RPB2–7R 5′-CCCATGGCTGACTGGTAA-3′ for *RPB2* and BT2a 5′-GGAAACCAAATTGGTGCTGCCTTC-3′ and BT2b 5′-ACCCTCTGTGTAGTGACCCTTGGC-3′ for *BT2* amplification.

Amplified products were purified using the NucleoSpin extract II kit (Macherey-Nagel) and commercially sequenced in both directions. Sequencing analysis was performed using the BioEdit sequence Alignment Editor (version 7.1.11) and the sequences were trimmed as previously described by Bernhardt et al.[21] The sequence type (ST) of each isolate was defined by the combination of alleles identified at the different loci studied. All obtained data were deposited in the Genbank database and submitted to the Fungal MLST Database.

Results

Species identification within the *S. apiospermum* complex

TUB sequencing was used for precise species identification within the *S. apiospermum* complex. *Scedosporium boydii* was the most common species with a total number of 36 isolates recovered from 15 sputum samples collected from patients P1, P3, P7, and P9, whereas *S. apiospermum* was identified from three patients (P4, P5, and P8), representing 21 isolates (Table 1). All isolates from patient P6 were identified by TUB sequencing as *S. aurantiacum,* whereas the two isolates studied for patient P2 were reassigned to the *P. ellipsoidea* subgroup of *S. boydii*. *Scedosporium minutisporum* was not recovered from the nine CF patients studied here, and none of the patients was colonized by two distinct species.

Performances of Mold and *Aspergillus* rep-PCR assays and criteria for strain comparison

rep-PCR was conducted on our set of isolates using two primer kits designed to amplify all mold species (Mold DNA fingerprinting primer kit) or more specifically *A. fumigatus* (*Aspergillus*-specific DNA fingerprinting primer kit), and from the obtained electrophoretic profiles, the isolates were categorized as indistinguishable, closely related or different according to the interpretative criteria provided by the manufacturer. An example of isolates sharing the same electrophoretic profile and therefore belonging to the same rep-PCR genotype is presented in Figure 1A with *S. apiospermum* IHEM 14762 and 14763 isolates obtained from the same sputum sample from patient P8. Figure 1B shows an example of closely related isolates, that is, differing only by 1 or 2 peaks, with *S. apiospermum* IHEM 14273 and 15149 isolates from patients P4 and P5, respectively, and an example of isolates with -electrophoretic profiles differing by at least three peaks, therefore belonging to different genotypes, is shown in Figure 1C, with *S. apiospermum* IHEM 14270 and 15146 isolates recovered from patients P4 and P8, respectively.

The use of the Mold primer kit always resulted in the detection of a greater number of electrophoretic bands as shown for *S. boydii* IHEM 14636 and *S. aurantiacum* IHEM 15461 isolates presented as typical examples (Fig. 2). Moreover, this kit was more discriminant than the *Aspergillus*-specific primer kit. Seven different profiles were obtained for the whole set of clinical isolates using the *Aspergillus*-specific primer kit, compared to 10 genotypes identified with the Mold primer kit (Table 1). Finally, as no *S. minutisporum* isolates were detected among the clinical

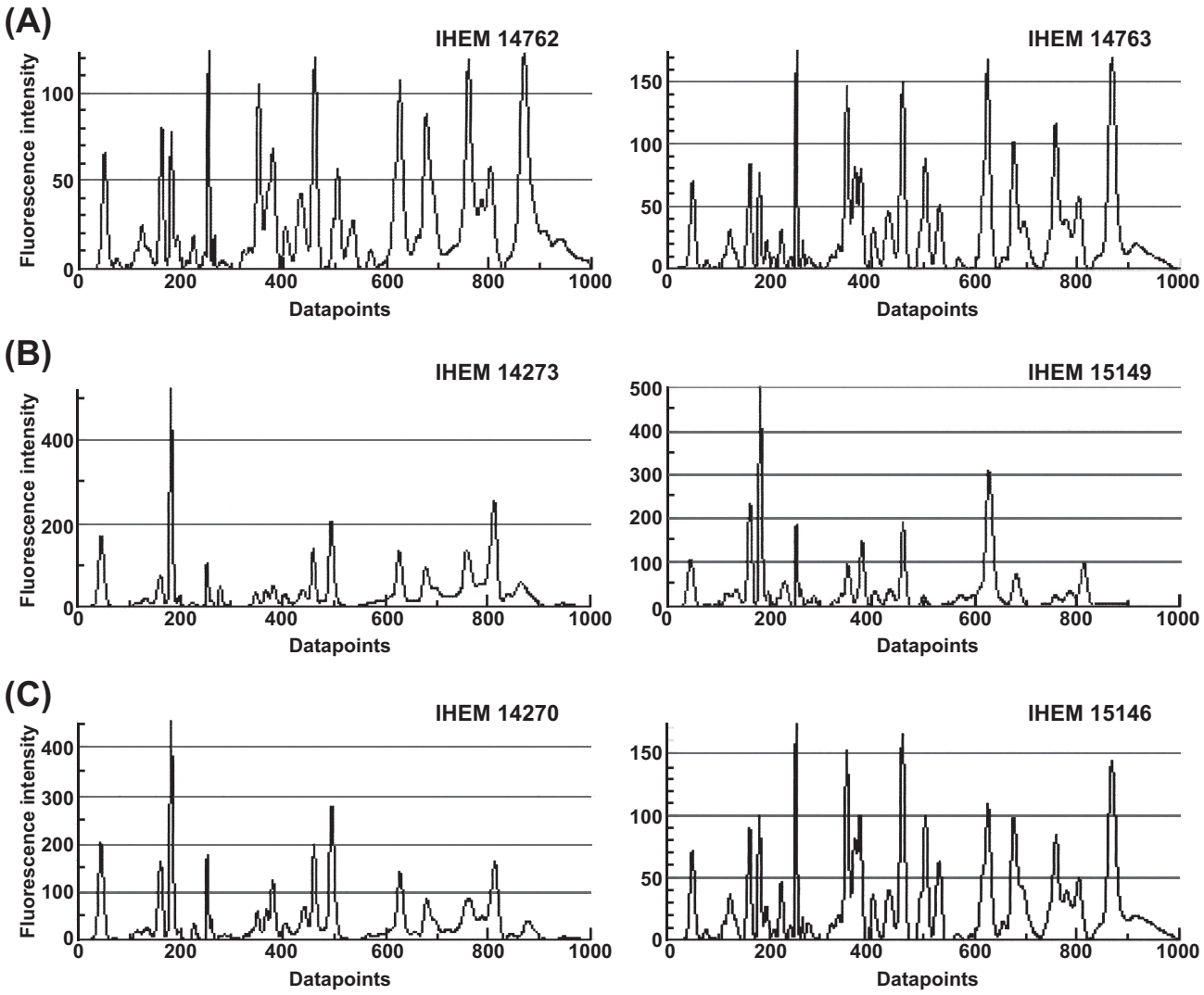


Figure 1. Typical examples of electrophoretic profiles obtained by rep-PCR using the Mold DNA fingerprinting primer kit. (**A**) identical profiles (*S. apiospermum* IHEM 14762 and 14763 isolates); (**B**) closely related profiles (*S. apiospermum* 14273 and 15149 isolates); and (**C**) distinct profiles (*S. apiospermum* IHEM 14270 and 15146 isolates).

isolates studied here, an environmental isolate was analyzed by rep-PCR, which clearly differentiated this isolate from the others.

In addition, data were analyzed using the DiversiLab software, which generates dendrograms showing fingerprint similarities between the isolates. All isolates from the same species were clustered in the dendrogram generated from data obtained with the Mold primer kit (Fig. 3A) as well as using the *Aspergillus*-specific primer kit (Fig. 3B). With both primer kits, two groups of isolates were distinguished within *S. apiospermum sensu stricto*. However, compared to the Mold primer kit, the use of the *Aspergillus*-specific primer kit separated the species in a slightly different order. For instance, whereas the two *P. ellipsoidea* isolates were clustered with the other *S. boydii* isolates with the Mold primer kit, they were clearly separated from the other *S. boydii* isolates when using the *Aspergillus*-specific primer kit (Fig. 3B).

Comparison of rep-PCR wit MLST and RAPD for strain differentiation

Compared to the *Aspergillus*-specific primer kit, the use of the mold primer kit provided results more consistent with those obtained by RAPD and MLST (Table 1). For example, the *Aspergillus* rep-PCR assay could not differentiate the *S. boydii* isolates, always producing for these isolates the same electrophoretic profile. In contrast, results obtained using the Mold primer kit were in close agreement with those obtained by MLST. Using this kit, rep-PCR showed

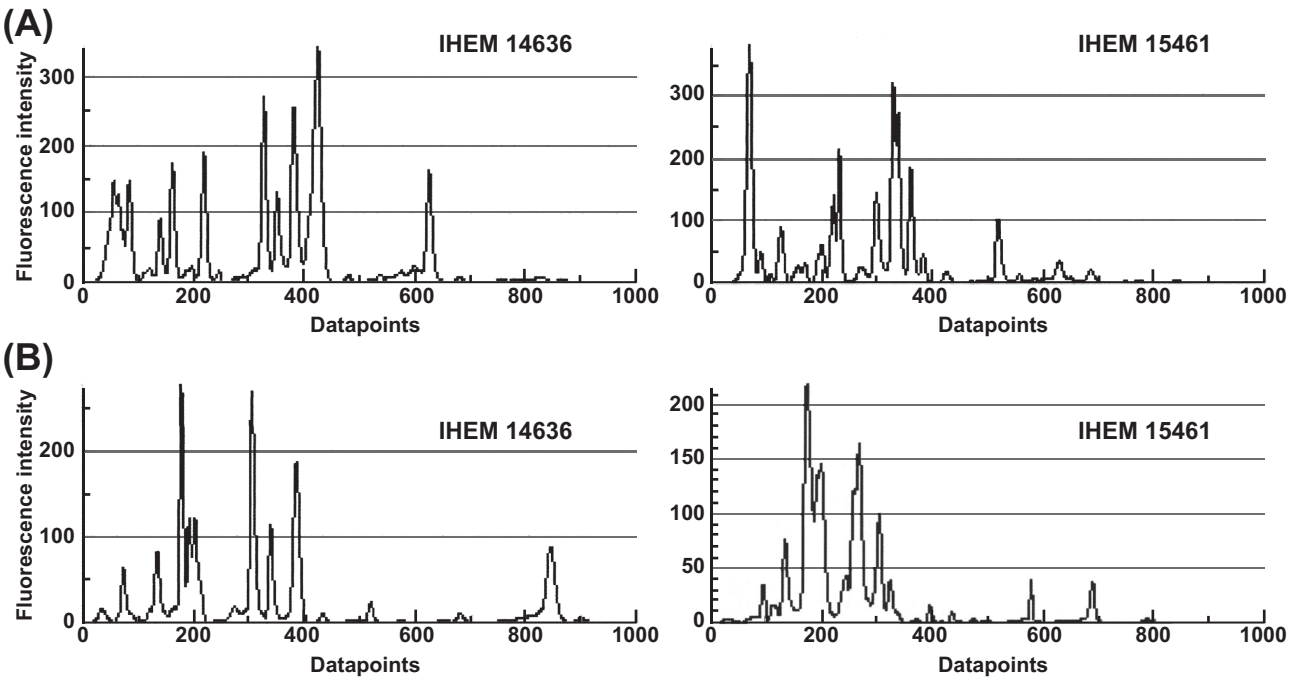


Figure 2. Comparison of electrophoretic profiles generated using the Mold DNA fingerprinting primer kit (**A**) or with the *Aspergillus*-specific fingerprinting primer kit (**B**). Electrophoretic profiles obtained for *S. boydii* IHEM 14636 and *S. aurantiacum* IHEM 15461 isolates are shown as examples.

that patients P3, P7 and P9 were each colonized by a single genotype, differing from one patient to another, as demonstrated by RAPD and MLST. Conversely, while three genotypes were identified by RAPD for patient P1 with a largely dominant genotype (11 isolates) associated with two other genotypes very close to the dominant genotype and found only once each, single electrophoretic profile and sequence type were seen by rep-PCR and MLST for all isolates from this patient.

Regarding *S. apiospermum sensu stricto*, patients P4 and P5 were also colonized each by a single strain as suggested by rep-PCR, which was confirmed by RAPD and MLST. By contrast, two distinct rep-PCR genotypes were found successively in sputum samples from patient P8 using the Mold primer kit, with at first a transient carriage of Mold rep-PCR genotype 10 unable to establish within the respiratory tract, followed by a chronic colonization of the airways by Mold rep-PCR genotype 11, and a similar evolution was revealed by RAPD and MLST. Likewise, three distinct electrophoretic profiles were seen with the *Aspergillus*-specific primer kit for the four isolates belonging to MLST sequence type 38. Moreover, using the *Aspergillus*-specific primer kit, the same electrophoretic profile was seen for patient P5's isolates and most of the isolates from patient P8, whereas the use of the Mold primer kit clearly differentiated the isolates from these two patients, in agreement with the results obtained by RAPD and MLST.

Four *S. aurantiacum* isolates originating from the same sputum sample from patient P6 were also studied. Whatever the rep-PCR primer kit used, these isolates shared the same electrophoretic profile and therefore belonged to the same genotype, in agreement with the results obtained by RAPD and MLST.

Moreover, as they belonged to the *P. ellipsoidea* subgroup of *S. boydii*, the two isolates studied for patient P2 were not analyzed by MLST. Nevertheless, in agreement with results from RAPD, both isolates were shown to belong to the same genotype by rep-PCR.

Discussion

In most cases, the airway colonization by species of the *S. apiospermum* complex in CF is asymptomatic.[3,4] However, even in the absence of an obvious respiratory infection, careful attention is required in case of chronic colonization of the airways, because of the propensity of these fungi to disseminate in lung transplant recipients[9–11] and of their primary resistance to echinocandins and very low susceptibility to current triazole drugs.[12–14] Additionally, there is now accumulating evidence indicating that persistent carriage of fungi in CF patients without clinical signs of respiratory infection is a risk factor for a progressive worsening of the lung function.[27–29] Compared to non-colonized patients and transient carriers, the chronic colonization of

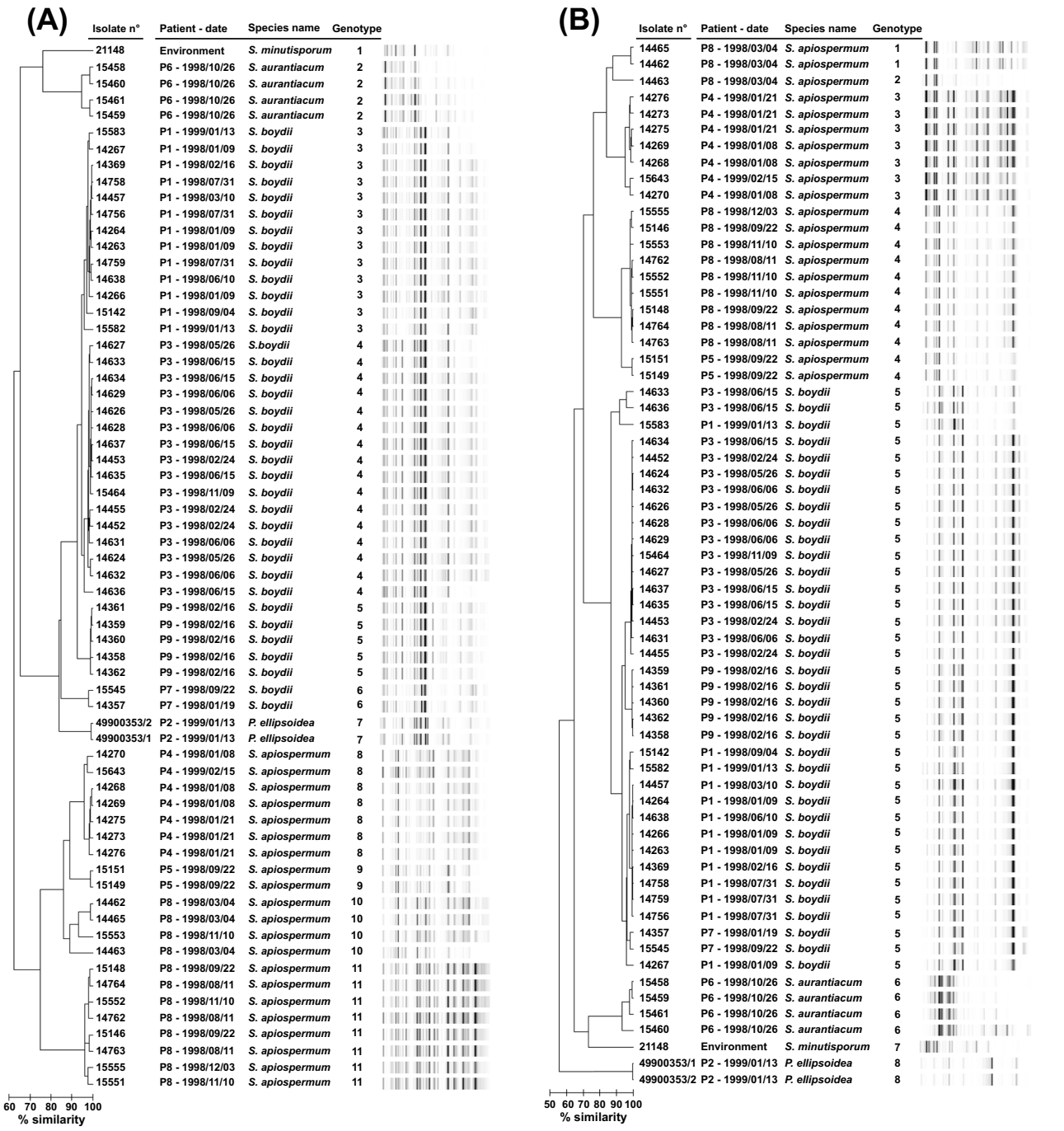


Figure 3. Phylogenetic tree generated from the analysis of data obtained with the Mold DNA fingerprinting primer kit (**A**) or with the *Aspergillus*-specific fingerprinting primer kit (**B**). Data were analyzed with the unweighted pair group method with arithmetic mean (UPGMA) using the DiversiLab Healthcare software and genotypes were numbered according to their order in the phylogenetic trees. The type strain of *S. minutisporum* (IHEM strain 21148, also called FMR 4072) was included in the panel of isolates studied.

the airways by *A. fumigatus* or *Candida albicans* is an independent risk factor for a decline in the forced expiratory volume in one second (FEV_1) and hospital-treated exacerbations in CF.[27–29] Therefore an early and accurate detection of fungi appears essential for appropriate treatment.

Since the works of Gilgado et al.[15–17], *S. apiospermum* is considered a species complex comprising five distinct species, four of them having been identified in the CF context: *S. apiospermum sensu stricto*, *S. boydii*, *S. aurantiacum*, and *S. minutisporum*.[19] The precise species identification within the *S. apiospermum* complex is

important not only for a better knowledge of the epidemiology of airway colonization / clinical infections but also because of differences between species in their susceptibility to antifungals and pathogenicity.[12–14,30] However, precise species identification is not possible on a morphological basis. Several molecular methods allowing the detection of the *S. apiospermum* complex from respiratory secretions and, for some of them, direct species identification, have been described such as quantitative real-time PCR, PCR-based reverse line blot hybridization and loop-mediated isothermal amplification.[31] Nevertheless, these methods are not commercially available and therefore uneasy to use in routine practice. A rapid and accurate species differentiation from positive cultures may be reached by matrix-assisted LASER desorption ionization time-of-flight / mass spectrometry, but until now validated databases for identification of these closely related species are available for the Andromas system only.[32,33] In addition, previous genotype studies on *A. fumigatus* or *Aspergillus terreus* in CF demonstrated that genotyping of multiple and sequential isolates is required to distinguish between a regular, but transient carriage of distinct genotypes, which is not clinically relevant, from a chronic colonization of the airways, which indicates growth and proliferation of the fungus in the CF airways, and therefore its contribution to the inflammatory reaction and to a clinical or functional worsening.[34,35] However, none of the above mentioned methods allows this strain delineation.

In the present study, rep-PCR permitted precise species identification of clinical isolates from CF patients within the *S. apiospermum* complex as previously shown for the two major species of this complex, that is, *S. apiospermum sensu stricto* and *S. boydii*.[36] In addition we also demonstrated that this method may be used for strain delineation within this species complex, at least for the two major species. Species identification and strain delineation were found easier using the Mold DNA fingerprinting primer kit than the *Aspergillus*-specific primer kit, which always produced lower peak numbers and was less discriminant. These two kits never were compared in previous studies performed on evaluation of the performances of rep-PCR for identification and/or strain differentiation for fungi. Apart from the work of Healy et al.,[23] the Mold DNA fingerprinting primer kit was the only one used in studies on filamentous fungi.[24,36] Nevertheless, it is likely that the primers included in the *Aspergillus*-specific kit are too distant from the target DNA sequences on other fungal species, and one may recommend the use of the Mold kit for studies on non-*Aspergillus* filamentous fungi.

Interestingly, rep-PCR data were consistent with species identification and a close agreement was shown for strain differentiation between rep-PCR and MLST. Dendrogram analysis of the data generated with the Mold primer kit revealed a clustering very close to that reported by Gilgado et al.[15] Interestingly, species clustered in a different order using the *Aspergillus*-specific primer kit, with the two *P. ellipsoidea* isolates clearly separated from the other *S. boydii* isolates. rep-PCR thus provides new experimental data reviving the debate about the species status of *P. ellipsoidea*.[18]

From previous analysis of our set of isolates by RAPD, it was suggested that CF patients are usually colonized by a single genotype conserved over time despite the antifungal therapy.[20] Whatever the species within the *S. apiospermum* complex, a single genotype different from one patient to another was continuously present in almost all patients during their follow-up, and this was confirmed by rep-PCR and MLST. Interestingly, distinct genotypes were found during the follow-up in a few cases, with a largely dominant genotype responsible for a chronic colonization of the airways and one or two others unable to establish within the respiratory tract. Previous studies performed on the molecular epidemiology of the airway colonization by *A. fumigatus* or *A. terreus* in CF also showed some genotypes unable to chronically colonize the respiratory tract and disappearing in later samples for a genotype better adapted to the CF lungs.[34,35]

Genotyping is also crucial for epidemiological purposes. Species of the *S. apiospermum* complex are worldwide-distributed filamentous fungi, but the origin of patients' contamination is still unknown. An environmental study performed in Austria and the Netherlands showed that *S. apiospermum* is the most frequent species in industrial areas, city parks and playgrounds and agricultural areas.[37] In contrast, *S. boydii* was found the major species in a French cohort of CF patients colonized by the *S. apiospermum* complex.[19] rep-PCR, which allows both species identification and genotype analysis, should facilitate the comparison of clinical and environmental isolates and thus the identification of the origin of patients' contamination.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

References

1. George AM, Jones PM, Middleton PG. Cystic fibrosis infections: treatment strategies and prospects. *FEMS Microbiol Lett* 2009; **300**: 153–164.
2. Cohen-Cymberknoh M, Shoseyov D, Kerem E. Managing cystic fibrosis: strategies that increase life expectancy and improve quality of life. *Am J Respir Crit Care Med* 2011; **183**: 1463–1471.
3. Pihet M, Carrère J, Cimon B et al. Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis–a review. *Med Mycol* 2009; **47**: 387–397.
4. Cimon B, Carrère J, Vinatier JF et al. Clinical significance of *Scedosporium apiospermum* in patients with cystic fibrosis. *Eur J Clin Microbiol Infect Dis* 2000; **19**: 53–56.
5. Masoud-Landgraf L, Badura A, Eber E et al. Modified culture method detects a high diversity of fungal species in cystic fibrosis patients. *Med Mycol* 2014; **52**: 179–186.
6. Horré R, Marklein G, Siekmeier R et al. Selective isolation of *Pseudallescheria* and *Scedosporium* species from respiratory tract specimens of cystic fibrosis patients. *Respiration* 2009; **77**: 320–324.
7. Harun A, Gilgado F, Chen SC et al. Abundance of *Pseudallescheria/Scedosporium* species in the Australian urban environment suggests a possible source for scedosporiosis including the colonization of airways in cystic fibrosis. *Med Mycol* 2010; **48**(Suppl. 1): S70–S76.
8. Vázquez-Tsuji O, Campos Rivera T, Rondán Zárate A et al. Endobronchitis by *Scedosporium apiospermum* in a child with cystic fibrosis. *Rev Iberoam Micol* 2006; **23**: 245–248.
9. Symoens F, Knoop C, Schrooyen M et al. Disseminated *Scedosporium apiospermum* infection in a cystic fibrosis patient after double-lung transplantation. *J Heart Lung Transplant* 2006; **25**: 603–607.
10. Morio F, Horeau-Langlard D, Gay-Andrieu F et al. Disseminated *Scedosporium/Pseudallescheria* infection after double-lung transplantation in patients with cystic fibrosis. *J Clin Microbiol* 2010; **48**: 1978–1982.
11. Hirschi S, Letscher-Bru V, Pottecher J et al. Disseminated *Trichosporon mycotoxinivorans*, *Aspergillus fumigatus*, and *Scedosporium apiospermum* coinfection after lung and liver transplantation in a cystic fibrosis patient. *J Clin Microbiol* 2012; **50**: 4168–4170.
12. Gilgado F, Serena C, Cano J et al. Antifungal susceptibilities of the species of the *Pseudallescheria boydii* complex. *Antimicrob Agents Chemother* 2006; **50**: 4211–4213.
13. Alastruey-Izquierdo A, Cuenca-Estrella M, Monzón A et al. Prevalence and susceptibility testing of new species of *Pseudallescheria* and *Scedosporium* in a collection of clinical mold isolates. *Antimicrob Agents Chemother* 2007; **51**: 748–751.
14. Lackner M, de Hoog GS, Verweij PE et al. Species-specific antifungal susceptibility patterns of *Scedosporium* and *Pseudallescheria* species. *Antimicrob Agents Chemother* 2012; **56**: 2635–2642.
15. Gilgado F, Cano J, Gené J et al. Molecular phylogeny of the *Pseudallescheria boydii* species complex: proposal of two new species. *J Clin Microbiol* 2005; **43**: 4930–4942.
16. Gilgado F, Cano J, Gené J et al. Molecular and phenotypic data supporting distinct species statuses for *Scedosporium apiospermum* and *Pseudallescheria boydii* and the proposed new species *Scedosporium dehoogii*. *J Clin Microbiol* 2008; **46**: 766–771.
17. Gilgado F, Gené J, Cano J et al. Heterothallism in *Scedosporium apiospermum* and description of its teleomorph *Pseudallescheria apiosperma* sp. nov. *Med Mycol* 2010; **48**: 122–128.
18. Lackner M., de Hoog S.G., Yang L. et al. Proposed nomenclature for *Pseudallescheria*, *Scedosporium* and related genera. *Fungal Diversity* 2014; **67**: 1–10.
19. Zouhair R, Rougeron A, Razafimandimby B et al. Distribution of the different species of the *Pseudallescheria boydii/Scedosporium apiospermum* complex in French patients with cystic fibrosis. *Med Mycol* 2013; **51**: 603–613.
20. Defontaine A, Zouhair R, Cimon B et al. Genotyping study of *Scedosporium apiospermum* isolates from patients with cystic fibrosis. *J Clin Microbiol* 2002; **40**: 2108–2114.
21. Bernhardt A, Sedlacek L, Wagner S et al. Multilocus sequence typing of *Scedosporium apiospermum* and *Pseudallescheria boydii* isolates from cystic fibrosis patients. *J Cyst Fibros* 2013; **12**: 592–598.
22. Wise MG, Healy M, Reece K et al. Species identification and strain differentiation of clinical *Candida* isolates using the DiversiLab system of automated repetitive sequence-based PCR. *J Med Microbiol* 2007; **56**: 778–787.
23. Healy M, Reece K, Walton D et al. Identification to the species level and differentiation between strains of *Aspergillus* clinical isolates by automated repetitive-sequence-based PCR. *J Clin Microbiol* 2004; **42**: 4016–4024.
24. Healy M, Reece K, Walton D et al. Use of the DiversiLab System for species and strain differentiation of *Fusarium* species isolates. *J Clin Microbiol* 2005; **43**: 5278–5280.
25. Hammarskjöld F, Mernelius S, Andersson RE et al. Possible transmission of *Candida albicans* on an intensive care unit: genotype and temporal cluster analyses. *J Hosp Infect* 2013; **85**: 60–65.
26. Vandeputte P, Ghamrawi S, Rechenmann M et al. Draft genome sequence of the pathogenic fungus *Scedosporium apiospermum*. *Genome Announc.* 2014; **2**: e00988–14.
27. Amin R, Dupuis A, Aaron SD et al. The effect of chronic infection with *Aspergillus fumigatus* on lung function and hospitalization in patients with cystic fibrosis. *Chest* 2010; **137**: 171–176.
28. Chotirmall SH, O'Donoghue E, Bennett K et al. Sputum *Candida albicans* presages FEV_1 decline and hospital-treated exacerbations in cystic fibrosis. *Chest* 2010; **138**: 1186–1195.
29. Fillaux J, Brémont F, Murris M et al. Assessment of *Aspergillus* sensitization or persistent carriage as a factor in lung function impairment in cystic fibrosis patients. *Scand J Infect Dis* 2012; **44**: 842–847.
30. Gilgado F, Cano J, Gené J et al. Different virulence of the species of the *Pseudallescheria boydii* complex. *Med Mycol* 2009; **47**: 371–374.
31. Lu Q, Gerrits van den Ende AH, Bakkers JM et al. Identification of *Pseudallescheria* and *Scedosporium* species by three molecular methods. *J Clin Microbiol* 2011; **49**: 960–967.

32. DelChierico F, Masotti A, Onori M et al. MALDI-TOF MS proteomic phenotyping of filamentous and other fungi from clinical origin. *J Proteomics* 2012; **75**: 3314–3330.
33. Sitterlé E, Giraud S, Leto J et al. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and accurate identification of *Pseudallescheria/Scedosporium* species. *Clin Microbiol Infect* 2014; **20**: 929–935.
34. Cimon B, Symoens F, Zouhair R et al. Molecular epidemiology of airway colonisation by *Aspergillus fumigatus* in cystic fibrosis patients. *J Med Microbiol* 2001; **50**: 367–374.
35. Rougeron A, Giraud S, Razafimandimby B et al. Different colonization patterns of *Aspergillus terreus* in patients with cystic fibrosis. *Clin Microbiol Infect* 2014; **20**: 327–333.
36. Steinmann J, Schmitt D, Buer J et al. Discrimination of *Scedosporium prolificans* against *Pseudallescheria boydii* and *Scedosporium apiospermum* by semiautomated repetitive sequence-based PCR. *Med Mycol* 2011; **49**: 475–483.
37. Kaltseis J, Rainer J, de Hoog GS. Ecology of *Pseudallescheria* and *Scedosporium* species in human-dominated and natural environments and their distribution in clinical samples. *Med Mycol* 2009; **47**: 398–405.