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Characterization of *Scedosporium prolificans* Clinical Isolates by Randomly Amplified Polymorphic DNA Analysis

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Fingerprinting by randomly amplified polymorphic DNA (RAPD) analysis was used to differentiate *Scedosporium prolificans* isolates. A total of 59 arbitrary primers were screened with six unrelated *S. prolificans* isolates, and a panel of 12 primers was selected. The 12 primers were then used to detect DNA polymorphisms among 17 *S. prolificans* isolates from 11 patients with systemic *S. prolificans* infections diagnosed in three hospitals located in geographically different areas of Spain. Eight patients were diagnosed with *S. prolificans* infection in a single institution over a 6-year period, and two other patients were diagnosed with *S. prolificans* infection in a different hospital over a 1-year period. No single primer allowed for the discrimination of all the isolates from different patients, but this was possible by combining the RAPD patterns from three primers (UBC 701, AB1.08, and AB1.11 or UBC 701, AB1.08, and UBC 707). However, multiple isolates from the same patient were identical. In this study, we also compared a visual method and a computerized method for the analysis of the RAPD patterns. Both methods were satisfactory and gave few discordances, but given the advantages and disadvantages of each method, both systems should be used together. RAPD analysis provided a fast and economical means of typing *S. prolificans* isolates, with a high level of discrimination among unrelated isolates. Typing by RAPD analysis confirmed that the *S. prolificans* infections were epidemiologically unrelated.

Scedosporium prolificans, which was previously named *Scedosporium inflatum* (6), is a dematiaceous hyphomycete that causes focally invasive infections in nonimmunocompromised patients and systemic infections in immunocompromised patients (23, 24). The infections in the immunocompromised patients are usually diagnosed late in the course of the infection, and they are difficult to treat, since the fungus is highly resistant in vitro to all known antifungal agents (23). Despite this resistance, the clearance of the infection following administration of fluconazole or granulocyte colony-stimulating factor has recently been reported in two patients (3, 14).

Very little is known about the epidemiology, mode of transmission, or pathogenesis of *S. prolificans* infections. The natural habitat of *S. prolificans* is unknown, but it is supposed to be ubiquitous in soil and it has been isolated from soil from potted plants in a hospital (20). Infections are supposed to be acquired by inhalation or inoculation of spores, and they have been described in several countries (1, 11, 13, 14, 16, 17, 21, 23, 24). Sixteen cases of *S. prolificans* infection have been described in Spain (1, 3, 4, 10, 11, 21), and eight of them were reported in two institutions (1, 21). Up to now, no typing method has been described for *S. prolificans*. However, as with other fungi, use of typing methods would be helpful for epidemiological analysis of infections caused by *S. prolificans*. Different typing methods have been used for strain delineation in fungi, including both phenotypic and genotypic methods (2, 7, 9). In this study we examined the use of randomly selected primers to distinguish among isolates of *S. prolificans* and to characterize the isolates from a single institution.

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MATERIALS AND METHODS

Strains. Seventeen *S. prolificans* isolates were obtained from 11 patients with *S. prolificans* infections diagnosed in three hospitals located in different parts of Spain (Table 1). The infections appeared to be unrelated epidemiologically. The isolates were identified by both colonial morphology on potato-dextrose agar (prepared from raw components) and conidial morphology on potato-dextrose agar slide cultures (18). Colonial morphology was examined after incubation for 14 days at 25°C. The identity of the isolate from patient IV was confirmed by Colin K. Campbell at the Mycological Reference Laboratory, Public Health Laboratory Service, Bristol, England. This isolate has been included in the National Collection of Pathogenic Fungi (NCPF 2799) and was used as a control. One *Scedosporium apiospermum* isolate from a leukemic patient was also included to study the ability of the primers to discriminate between the two species. Identification of this isolate was also confirmed by Colin K. Campbell, and it has been included in the National Collection of Pathogenic Fungi and has been given the designation 7174. The isolates were maintained at room temperature on slants containing 20 g of glucose, 10 g of yeast extract, and 20 g of agar per liter.

Isolation of genomic DNA. For DNA isolation, fungi were cultured in Sabouraud dextrose broth for 3 days at 37°C under shaking at 100 rpm. The mycelia were collected in a tube and were washed twice with sterile distilled water. Genomic DNA preparation and randomly amplified polymorphic DNA (RAPD) analysis were performed by a modification of the method described for yeasts by Lehmann et al. (9). Briefly, glass beads were added to each tube to break the mycelia by vigorous beating in 250 μl of extraction buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA) mixed with 250 μl of phenol-chloroform-isoamyl alcohol (25:24:1; vol/vol/vol). Following the addition of 250 μl of TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and centrifugation (13,000 × *g*, 5 min) to remove the debris, the aqueous supernatant was extracted again with phenol-chloroform-isoamyl alcohol, and the nucleic acids were precipitated by the addition of ethanol. Genomic DNA was further purified by treatment of the preparation with 0.075 μg of RNase A (Boehringer Mannheim, Barcelona, Spain) per ml and a new precipitation with ethanol. The purified DNA was diluted to 2 ng/μl for the RAPD analyses.

TABLE 1. Origins of *S. prolificans* isolates examined in the study

Patient	Geographic location in Spain	Source of cultures	Date of isolation (mo.day.yr)	Isolate no.	Reference
I	Vizcaya	Blood	1.16.1990	3669	8
II	Valencia	Blood	5.15.1990	3569	8
III	Valencia	Blood	3.12.1989	3668	16
IV	Santander	Blood	2.22.1991	NCPF 2799	19
V	Santander	Bronchial aspirate	5.19.1993	93/39833	24
VI	Santander	BAL[a]	2.01.1995	95207	NA[b]
VII	Santander	Sputum	1.19.1996	96026	NA
VIII	Santander	CSF[c]	3.25.1993	93252	24
		CSF	3.30.1993	93253	
IX	Santander	Blood	12.21.1993	94001	24
		Lung biopsy	12.26.1993	94004	
X	Santander	Sputum	1.09.1996	96011	NA
		Sputum	1.16.1996	96025	NA
XI	Santander	Sputum	11.18.1993	93394	NA
		Sputum	11.29.1993	93395	NA
		BAL	12.03.1993	93398	NA
		Sputum	12.22.1993	94007	NA

[a] BAL, bronchoalveolar lavage.
[b] NA, not applicable.
[c] CSF, cerebrospinal fluid.

RAPD analysis. PCRs were performed in 0.5-ml microcentrifuge tubes by the method described by Sullivan et al. (19). The primers used in this study were AB1.01, 5′-GTTTCGCTCC-3′; AB1.08, 5′-GTCCACACGG-3′; AB1.11, 5′-GTAGACCCGT-3′; AB1.18, 5′-CCACAGCAGT-3′; AB1.20, 5′-GGACCCTTAC-3′; UBC-701, 5′-CCCACAACCC-3′; UBC-703, 5′-CCAACCACCC-3′; UBC-706, 5′-GGTGGTTGGG-3′; UBC-707, 5′-CCCAACACCC-3′; UBC-717, 5′-CCCACACCCA-3′; UBC-721, 5′-CCCACCCACA-3′; and UBC-722, 5′-CCTCTCCCTC-3′. The AB1 primers were purchased from Advanced Technologies Ltd. (Leatherhead, United Kingdom), and the UBC primers were purchased from J. R. Hobbs, University of British Columbia (Vancouver, British Columbia, Canada).

Each PCR mixture contained 10 ng of genomic DNA; 2.5 μl of 10× reaction buffer [160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, (pH 8.8), 0.1% Tween 20]; 20 pmol of each oligonucleotide primer; 200 μM (each) dATP, dGTP, dTTP, and dCTP; 3 mM $MgCl_2$; and 1.2 U of DNA polymerase (BIOTAQ DNA polymerase; Bioline, London, England). The volume was made up to 25 μl with double-distilled water. The mixtures were overlaid with 25 μl of mineral oil (Sigma Chemical, St. Louis, Mo.). The DNA was amplified in a Linus FTS-1 thermal cycler (Cultek, Madrid, Spain) with an initial cycle of 5 min of denaturation at 94°C; 40 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 36°C, and 2 min of extension at 72°C; and a final cycle of 1 min of extension at 72°C. The amplification products were analyzed by gel electrophoresis through 2% (wt/vol) agarose gels (Hispanlab, Alcobendas, Spain) with 0.5× Tris-borate-EDTA buffer (4.45 mM Tris hydrochloride, 4.45 mM boric acid, 25 mM EDTA [pH 8.0]) containing 20.0 ng of ethidium bromide per gel. Controls without the DNA template were used with the amplification mixture to test for contaminating DNA. The stabilities of the fingerprints during strain subculturing were assessed with two isolates, and they showed no variation. A 123-bp DNA ladder (Sigma) was used as a reference for the molecular sizes of the amplified DNA fragments.

Two methods were used to analyze the gels. In the computerized analysis, images of the gels were captured with a scanner (ScanJet IICX; Hewlett-Packard, Greeley, Colo.) at a resolution of 300 dots per in.^{2} and saved in a tagged image file format. The gels were analyzed with the software GelCompar, version 4.0 (Applied Maths, Kortrijk, Belgium), running in a 486DX4, 100-MHz personal computer. After conversion and normalization of the tracks, the RAPD patterns were saved in a database, and the relationships between isolates were compared by using the Pearson product-moment correlation coefficient and were visualized in dendrograms constructed from matrices of similarity values obtained by the unweighted pair-group method with the arithmetic averages algorithm (8). The index of similarity selected in each dendrogram to discriminate the isolates was dependent on the degree of similarity shown by the ladders run in each side of the gels (85 to 95%). In the visual analysis, the gels were inspected blindly with regard to the patient's identity by two independent observers. A difference of one or more discrete bands among the isolates was used as the criterion sufficient to designate different RAPD patterns.

RESULTS

Fifty-nine primers were initially chosen at random to obtain RAPD profiles for six unrelated isolates of *S. prolificans*. Most primers showed very similar patterns with these isolates, but 12 primers exhibited different degrees of discrimination among the isolates, and therefore, they were selected for subsequent studies with the full panel of isolates. The heterogeneity of the RAPD patterns obtained following use of some of the primers is presented in Fig. 1. No single primer allowed for the discrimination of all the isolates from different patients. When the inspection of the gels was made visually, primers UBC 701 and AB1.08 gave the most discriminatory results, allowing for the discrimination of isolates from eight and seven patients, respectively (Table 2). Other primers showed lower degrees of discrimination (Table 2). Since some of the primers studied showed complementary degrees of discrimination, the usefulness of combining the RAPD patterns obtained with different primers was studied. Thus, when the RAPD patterns, obtained with primers UBC 701, AB1.08, and AB1.11 or primers UBC 701, AB1.08, and UBC 707 were combined into a profile, it was possible to discriminate the isolates from the different patients studied (Table 2). By using these profiles, multiple isolates from the same patient had identical types, both for isolates obtained at different times and for isolates from different anatomical sites. For example, in patient XI, the isolates had been obtained over 34 days, while in patient IX, the isolates were obtained from blood and from a lung biopsy specimen.

The computerized analysis of the gels allowed for an easy comparison of the different gel tracks and the construction of dendrograms. In general, it was less discriminatory than visual inspection, since by use of an index of similarity of between 85 and 95%, only four types of RAPD patterns were observed with primer UBC 701 among the 17 *S. prolificans* isolates studied. Primer AB1.08 produced six types of RAPD patterns, but the other primers had lower discriminatory abilities. As in the visual inspection of the gels, the association of the computerized RAPD patterns obtained with three primers (AB1.20, UBC 701, and AB1.08) allowed for the discrimination of 11 isolates from different patients (Table 2). However, in the computerized analysis of the gels, the two isolates from patient X gave different types of RAPD patterns and one of them was the same type as that of the isolate obtained from patient VII. Since the differences observed in the computerized RAPD patterns from the two isolates from patient X were due to primer AB1.08, a combination of primers excluding primer AB1.08 (AB1.20, UBC 703, and UBC 701) allowed for the differentiation of 10 isolates from different patients and resulted in no discrepancies in the RAPD patterns for multiple isolates from the same patient.

All primers studied allowed for the differentiation between *S. prolificans* and *S. apiospermum* since both species showed different amplification patterns. Examples of these differences are shown in Fig. 1A and D to F.

DISCUSSION

The PCR-based RAPD fingerprinting technique has shown great potential for identifying many microbial pathogens. This technique, unlike the traditional PCR analysis, does not re-

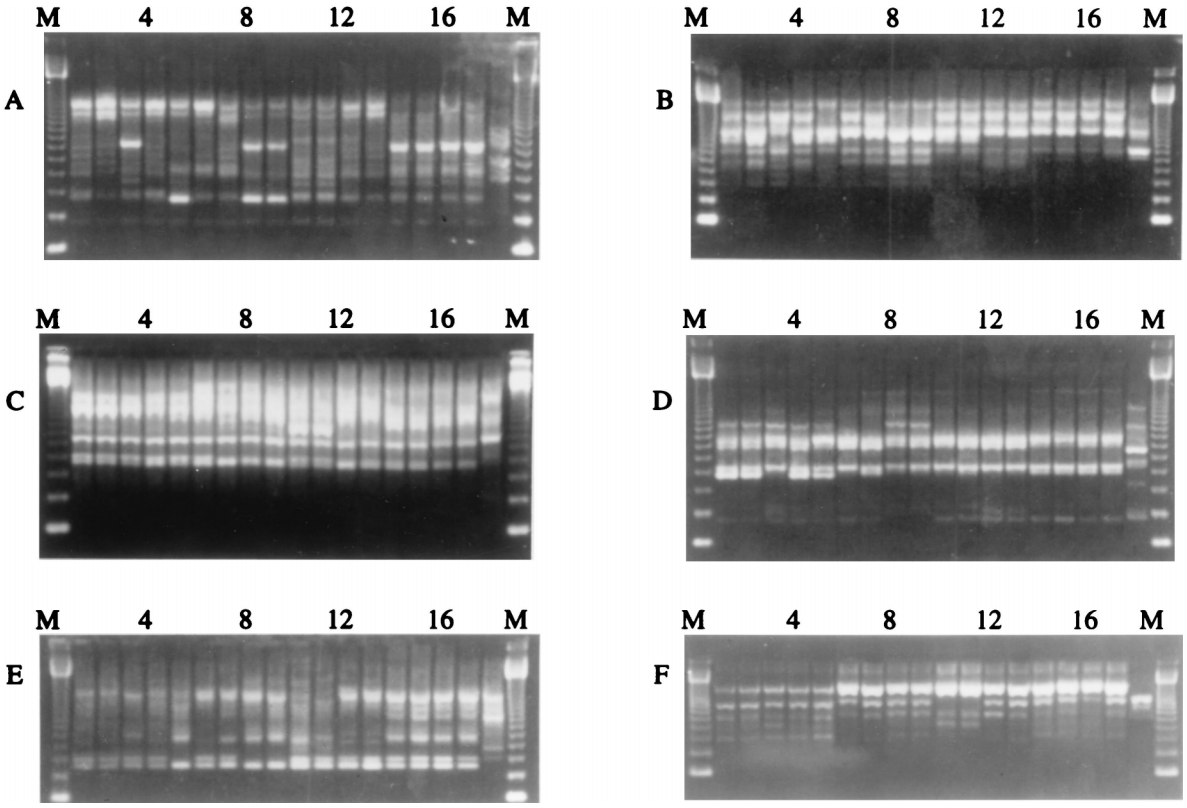

FIG. 1. RAPD patterns of *S. prolificans* and *S. apiospermum* isolates obtained with primers AB1.08 (A), UBC 701 (B), UBC 707 (C), AB1.11 (D), UBC 703 (E), and AB1.20 (F). Lanes 1 to 17, *S. prolificans* 3569, 3668, 3669, NCPF 2799, 93/93833, 95207, 96026, 93252, 93253, 94001, 94004, 96011, 96025, 93394, 93395, 93398, and 94007, respectively; lane 18, *S. apiospermum* NCPF 7174; lane M, 123-bp DNA ladder (123 to 4,182 bp in increments of 123 bp) as a molecular size marker.

quire any specific knowledge of the DNA sequences of the target organism, and therefore, the study of a large panel of primers is needed to select those capable of detecting DNA polymorphisms in the target organism. Despite its value in rapidly discriminating between individual isolates, the interlaboratory reproducibility is difficult due to a variety of factors (15, 22). Of the 59 primers studied in the work described in this report, 12 proved to be useful for the typing of *S. prolificans* isolates. This is in marked contrast to the results obtained by pulsed field gel electrophoresis, in which we were not able to resolve the *S. prolificans* chromosomes (data not shown). This was despite the fact that we used different DNA extraction procedures and a variety of electrophoretic conditions which had been useful for other filamentous fungi (12).

In this study, we have compared the results obtained by two methods of analysis of RAPD patterns. Overall, both methods were satisfactory and gave few discordances. When considering the ease of application, the computerized analysis of the gels is the method of choice, above all if a large number of isolates is being analyzed. However, it was slightly less discriminatory than the visual inspection. Computerized analysis allowed for the discrimination of the isolates from the 11 patients studied, but it gave different types of RAPD patterns for two isolates from a single patient. Conversely, the visual interpretation of the gels allowed for the discrimination of all the isolates from different patients, and multiple isolates from one patient were all found to be identical. However, this approach is more difficult to perform and takes more time than the computerized analysis. The reasons for the small number of discrepancies observed between the two methods may lie in the way that each method analyzes the RAPD profiles, since the computerized analysis compares densitometric arrays, while visual analysis is based on the differences in bands among the isolates. In fact, the differences between the computerized patterns from the two isolates from patient X are due to the intensities of the bands and not to a different number of bands in each pattern. Given the advantages and disadvantages of each method, we think that, when possible, both systems should be used together. Thus, the computerized analysis may be performed first, and then the results should be checked visually. The coincidence of both methods would reinforce the validity of the results.

One of the applications of RAPD PCR analysis is to establish whether isolates of a given microorganism from different patients or from a single patient are related. In our study, the results strongly suggest that each patient with systemic infection caused by *S. prolificans* was infected with a single different strain. This was demonstrated by finding, when using the combination of results obtained with three primers, the same RAPD patterns for all isolates from a single patient, regardless of their anatomic source or the time of sampling. In the case of the isolates from the same institution, our results suggest that each patient was infected with a different strain. Taking into consideration the fact that each patient came from different geographical locations and the fact that patients hospitalized in the Hospital de Valdecilla (Santander, Spain) were on different floors or in different buildings, it seems reasonable to think that infection occurred outside the hospital environment. This possibility has already been proposed for *Aspergillus fumigatus* infections (5) and in our study is supported by the inability to

TABLE 2. RAPD patterns obtained for the 17 *S. prolificans* isolates

Strain	Patient	Pattern obtained with the following primers[a]: AB1.13		UBC 705		AB1.01		AB1.18		UBC 706		UBC 717		UBC 722		AB1.20		AB1.11		UBC 703		UBC 707		UBC 721		AB1.08		UBC 701	
		V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C	V	C
3569	I	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	1
3668	II	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	2	A	1	A	1	A	1	A	1	B	1	B	2
3669	III	A	1	A	1	A	1	A	1	A	1	A	1	B	2	A	2	B	2	B	2	A	1	B	1	C	2	C	3
NCPF 2799	IV	A	1	A	1	A	1	A	1	A	1	A	1	A	1	A	2	A	1	A	1	A	1	A	1	A	1	B	1
93/39833	V	A	1	A	1	B	1	B	1	A	1	A	1	B	1	A	2	A	2	C	2	A	1	B	1	D	3	D	1
95207	VI	A	1	A	1	A	1	A	1	A	1	A	1	A	3	A	3	C	2	A	1	B	1	A	1	A	1	B	4
96026	VII	A	1	A	1	A	1	A	1	A	1	A	1	A	2	A	4	D	2	B	1	C	1	A	1	A	4	B	4
93252	VIII	A	1	A	1	A	1	A	1	A	1	A	1	B	2	A	4	B	3	C	2	C	1	B	1	E	5	E	2
93253	VIII	A	1	A	1	A	1	A	1	A	1	A	1	B	2	A	4	B	3	C	2	C	1	B	1	E	5	E	2
94001	IX	A	1	A	1	B	1	B	1	B	1	B	1	A	1	B	4	C	2	B	3	D	1	X	1	F	4	F	1
94004	IX	A	1	A	1	B	1	B	1	B	1	B	1	A	1	B	4	C	2	B	3	D	1	C	1	F	4	F	1
96011	X	A	1	A	1	A	1	A	1	A	1	A	1	A	2	A	4	C	2	D	1	B	1	D	1	A	4	G	4
96025	X	A	1	A	1	A	1	A	1	A	1	A	1	A	2	A	4	C	2	D	1	B	1	D	1	A	1	G	4
93394	XI	A	1	A	1	A	1	A	1	A	1	A	1	A	2	C	4	D	2	B	2	C	1	E	1	G	6	H	4
93395	XI	A	1	A	1	A	1	A	1	A	1	A	1	A	2	C	4	D	2	B	2	C	1	E	1	G	6	H	4
93398	XI	A	1	A	1	A	1	A	1	A	1	A	1	A	2	C	4	D	2	B	2	C	1	E	1	G	6	H	4
94007	XI	A	1	A	1	A	1	A	1	A	1	A	1	A	2	C	4	D	2	B	2	C	1	E	1	G	6	H	4
No. of types		1	1	1	1	2	1	2	1	2	1	2	1	2	3	3	4	4	3	4	3	4	1	5	1	7	6	8	4

[a] Visual analysis of the gel; C, computerized analysis of the gel.

isolate *S. prolificans* from the hospital environment, since tests of samples of air filters, ground, water, and the hands of medical personnel were negative (21).

In addition to the application for *S. prolificans* typing, all the primers studied allowed for the differentiation between *S. prolificans* and the closely related pathogen *S. apiospermum*. Among them, primers AB1.13 and UBC 705 appear to be the most appropriate for the differentiation between these two species, since the RAPD patterns of *S. apiospermum* were grossly different from those of the *S. prolificans* isolates.

Methods of typing *S. prolificans* isolated from infected patients may be useful in answering such questions as whether an isolate is responsible for infections occurring in the same institution and whether certain isolates are more likely to be recovered from patients with systemic infections. The results presented in this report, although limited by the number of isolates tested, revealed that RAPD PCR has a high discriminatory power and can successfully be applied to show the high level of genetic diversity that exists among *S. prolificans* isolates.

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