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Molecular Phylogeny of *Sporothrix schenckii*

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The pathogenic dimorphic fungus *Sporothrix schenckii* is the agent responsible for sporotrichosis, an important fungal infection with a worldwide distribution. Little is known about the population structure of *S. schenckii*, although recent molecular and phenotypic data seem to demonstrate that different genetic lineages exist within this species. The aim of this study was to determine, by sequence analysis of three protein coding loci (chitin synthase, β-tubulin, and calmodulin), whether this variability is due to species divergence or intraspecific diversity in *S. schenckii*. We included in the analysis 60 isolates (59 of clinical and 1 of environmental origin) of this species from a wide geographical range. DNA sequence data from the three nuclear regions were used in a phylogenetic analysis. The combined analysis of the three loci revealed the presence of three major clades, one grouping all of the European isolates, another with only Brazilian isolates, and the third with isolates from other South American countries and Africa. A total of 14 100% bootstrap-supported nodes were shown, 6 of them representing putative phylogenetic species. Our data also demonstrated that most of these species prevail in different geographical regions.

Sporothrix schenckii is a thermally dimorphic fungus responsible for sporotrichosis, a chronic granulomatous infection of the skin and subcutaneous tissues, although it can disseminate, affecting any organ of the human body (3). The infection is distributed worldwide, although it is more common in tropical and subtropical areas. Despite the clinical importance of *S. schenckii*, little is known about its basic biology and population structure. *S. schenckii* has its natural habitat in soil and plants, although it has been isolated from a variety of other sources (5, 14, 26). Recent molecular studies have demonstrated the existence of a high level of intraspecific variability and that isolates are mainly grouped according to their geographical origin (10, 11, 14, 16). More recently, on the basis of internal transcribed spacer (ITS) region sequence analysis, it has been suggested that more than one species could exist within *S. schenckii* (27). Travassos and Lloyd (26) and Ghosh et al. (6) had also found morphological and physiological differences between isolates of clinical origin and those from other sources. Differences in virulence between clinical and environmental strains were reported, but no correlation was found with the different clinical forms of sporotrichosis (5, 16, 25). In this study, we have used DNA sequence data from multiple loci to assess the extent of clonality within a group of clinical isolates of *S. schenckii* from different geographic regions. Several molecular studies based on multiple gene sequences have demonstrated the existence of numerous phylogenetic species within well-established morphological species (4, 7, 17). However, the definition of phylogenetic species is controversial (13). Taylor et al. (24) argued that in the recognition of phylogenetic species, one-gene ge-

nealogy is not enough and the concordance of multiple-gene genealogies is required. The Rio de Janeiro region has been affected by a long-lasting outbreak of cat-transmitted sporotrichosis (22). The aim of the present study was to determine if these infections are caused by a single, highly virulent strain or by strains with multiple origins.

MATERIALS AND METHODS

Fungal isolates. Sixty isolates morphologically identified as *S. schenckii* (59 from clinical sources and 1 from an environmental source) were included in this study (Table 1). Clinical isolates were provided by different reference culture collections (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; Facultat de Medicina i Ciències de la Salut, Reus, Spain; BCCM/IHEM Biomedical Fungi and Yeasts Collection, Brussels, Belgium; Instituto de Pesquisa Clínica Evandro Chagas, FIOCRUZ, Brazil; Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil; and Biological Resource Center, Chiba, Japan). Most of the clinical isolates from Brazil (18 isolates) and the environmental isolate were related to a long-lasting outbreak of cat-transmitted sporotrichosis (22). Clinical isolates were from 11 patients, and the environmental isolate was from the house dust of one of these patients. Isolates were stored on potato dextrose agar (Difco Laboratories, Detroit, Mich.) at 4 to 7°C and in slant cultures submerged in mineral oil at room temperature.

DNA extraction, amplification, and sequencing. DNA was extracted and purified directly from fungal colonies by following the Fast DNA kit protocol (Bio 101, Inc., Vista, Calif.) with the homogenization step repeated three times with a FastPrep FP120 instrument (Thermo Savant, Holbrook, N.Y.). After each homogenization, the sample was kept in ice for 10 min. DNA was quantified with GeneQuant *pro* (Amersham Pharmacia Biotech, Cambridge, England). Regions of the following nuclear genes were amplified by PCR: the chitin synthase (CHS) gene with primers CHS-79F and CHS-354R (2), the calmodulin (CAL) gene with degenerated primers CL1 and CL2A (17), and the β-tubulin (Bt2) gene with degenerated primers designed by us, i.e., Bt2-F [5′GG[CT]AACCA(AG)AT(ATC)GGTGC(CT)GC(CT)3′] and Bt2-R [5′ACCCTC(AG)GTGT AGTGACC CTTGGC3′] from primers Bt2a and Bt2b described by Glass and Donaldson (8). Amplifications were done by following the Ready-To-Go PCR bead protocol (Amersham Bioscience, Freiburg, Germany). For each reaction, we added 20 to 60 ng of DNA template and a 0.5 to 1 mM concentration of each primer in a total volume of 25 μl. The amplification program included an initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of denaturation at 95°C for 30 s, annealing for 1 min at 55°C (CHS) or 60°C (CAL and Bt2), and extension for 1 min at 72°C. A final extension step of 72°C for 7 min was included. The PCR

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TABLE 1. Isolates included in this study and their origins[a]

Isolate	Source	EMBL accession no.		
		CHS	Bt2	CAL
IPEC 16042	Skin lesion, Rio de Janeiro, Brazil	AM117416	AM116932	AM116885
IPEC 16243	Skin lesion, Rio de Janeiro, Brazil	AM117419	AM116957	AM116877
IPEC 16490	Skin lesion, Rio de Janeiro, Brazil	AM117417	AM116946	AM116899
IPEC 16503	Skin lesion, Rio de Janeiro, Brazil	AM114894	AM116941	AM116875
IPEC 16550	Skin lesion, Rio de Janeiro, Brazil	AM114893	AM116953	AM116892
IPEC 16864	Skin lesion, Rio de Janeiro, Brazil	AM117414	AM116951	AM116889
IPEC 16919	Skin lesion, Rio de Janeiro, Brazil	AM117407	AM116934	AM116898
IPEC 15572	Skin lesion, Rio de Janeiro, Brazil	AM117411	AM116955	AM116886
IPEC 16456	Skin lesion, Rio de Janeiro, Brazil	AM117406	AM116931	AM116897
IPEC 17943	Cerebrospinal fluid, Rio de Janeiro, Brazil	AM117410	AM116935	AM116878
IPEC 17307	Skin lesion, Rio de Janeiro, Brazil	AM117415	AM116949	AM116896
IPEC 17331	Skin lesion, Rio de Janeiro, Brazil	AM114899	AM116939	AM116880
IPEC 17608	Skin lesion, Rio de Janeiro, Brazil	AM117408	AM116937	AM116890
IPEC 17692	Skin lesion, Rio de Janeiro, Brazil	AM114897	AM116933	AM159127
IPEC 17920	Skin lesion, Rio de Janeiro, Brazil	AM114891	AM116938	AM116888
IPEC 17786	Skin lesion, Rio de Janeiro, Brazil	AM114898	AM116954	AM116884
IPEC 17521	Skin lesion, Rio de Janeiro, Brazil	AM114895	AM116952	AM116874
IPEC 17585	Skin lesion, Rio de Janeiro, Brazil	AM114892	AM116940	AM116887
IPEC 22487	Blood, Rio de Janeiro, Brazil	AM117409	AM116944	AM116882
IPEC 22493.1	Skin lesion, Rio de Janeiro, Brazil	AM117412	AM116945	AM116894
IPEC 22493.2	Skin lesion, Rio de Janeiro, Brazil	AM114900	AM116936	AM116883
IPEC 22496.4	Skin lesion, Rio de Janeiro, Brazil	AM117413	AM116950	AM116895
IPEC 22496.5	Skin lesion, Rio de Janeiro, Brazil	AM114896	AM116947	AM116879
IPEC 22582	Bone biopsy, Rio de Janeiro, Brazil	AM114889	AM116956	AM116891
IPEC 22593	Sputum, Rio de Janeiro, Brazil	AM114890	AM116942	AM116893
IPEC 22542/2	Disseminated sporotrichosis, Rio de Janeiro, Brazil	AM117418	AM116948	AM116881
IHEM 3774	Human sporotrichosis, Colombia	AM114873	AM116921	AM117447
IHEM 3787	South Africa	AM114870	AM116925	AM117435
IHEM 15477	Puncture of nodule, Bolivia	AM114878	AM116916	AM117444
IHEM 15486	Human sporotrichosis, Peru	AM114882	AM116929	AM117432
IHEM 15489	Human sporotrichosis, Peru	AM114883	AM116926	AM117430
IHEM 15499	Human sporotrichosis, Peru	AM114885	AM116928	AM117434
IHEM 15502	Human sporotrichosis, Peru	AM114884	AM116927	AM117427
IHEM 15503	Human sporotrichosis, Peru	AM114881	AM116930	AM117433
IHEM 15508	Human sporotrichosis, Peru	AM114875	AM116924	AM117443
IHEM 15511	Human sporotrichosis, Peru	AM114874	AM116917	AM117440
CBS 359.36[T]	NK	AM114872	AM116911	AM117437
NBRC 5984	NK	AM117424	AM116967	AM116900
NBRC 6072	NK	AM117420	AM116960	AM116904
NBRC 8158	NK	AM114868	AM116910	AM117438
FMR 8337	Domiciliary dust, Rio de Janeiro, Brazil	AM117421	AM116943	AM116876
FMR 8594	Hand lesion, Spain	AM114864	AM116965	AM116906
FMR 8595	Wrist lesion, Spain	AM117425	AM116959	AM116905
FMR 8596	Arm lesion, Spain	AM114866	AM116963	AM116902
FMR 8597	Leg lesion, Spain	AM117426	AM116964	AM116907
FMR 8598	Arm lesion, Spain	AM114867	AM116962	AM116903
FMR 8600	Face lesion, Spain	AM117422	AM116966	AM116908
FMR 8601	Finger lesion, Spain	AM114865	AM116958	AM116901
FMR 8602	Hand lesion, Spain	AM117423	AM116961	AM116909
FMR 8604	Cutaneous lymphatic lesion, Peru	AM114886	AM116914	AM117429
FMR 8605	Cutaneous lymphatic lesion, Peru	AM114869	AM116923	AM117442
FMR 8606	Cutaneous lymphatic lesion, Peru	AM114888	AM116913	AM117431
FMR 8607	Cutaneous lymphatic lesion, Peru	AM114887	AM116912	AM117428
FMR 8608	Cutaneous lymphatic lesion, Peru	AM114879	AM116919	AM117441
FMR 8609	Cutaneous lymphatic lesion, Peru	AM114880	AM116918	AM117439
FMR 8677	Disseminated sporotrichosis, Argentina	AM114871	AM116914	AM117436
FMR 8678	Skin biopsy, Argentina	AM114876	AM116920	AM117446
FMR 8679	Cutaneous lymphatic nodule, Argentina	AM114877	AM116922	AM117445
FMR 9034	Human sporotrichosis, São Paulo, Brazil	AM261684	AM261686	AM261688
FMR 9035	Human sporotrichosis, São Paulo, Brazil	AM261685	AM261687	AM261689

[a] Abbreviations: IPEC, Instituto de Pesquisa Clínica Evandro Chagas, FIOCRUZ, Rio de Janeiro, Brazil; IHEM, The BCCM/IHEM Biomedical Fungi and Yeasts Collection, Brussels, Belgium; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; NBRC, Biological Resource Center, Chiba, Japan; FMR, Facultat de Medicina i Ciències de la Salut, Reus, Spain; CHS, chitin synthase gene; Bt2, β-tubulin gene; CAL, calmodulin gene; T, type strain; NK, not known.

products were purified with a GFX™ PCR DNA purification kit (Pharmacia Biotech, Cerdanyola, Spain) and stored at −20°C until sequencing. PCR products were sequenced by using the above-mentioned primers and following the *Taq* DyeDeoxy Terminator cycle sequencing kit protocol (Applied Biosystems,

Gouda, The Netherlands). DNA sequencing reaction mixtures were analyzed on a 310 DNA sequencer (Applied Biosystems).

Phylogenetic analysis. The sequences were aligned with the ClustalX (version 1.81) computer program (25), followed by manual adjustments with a text editor.

TABLE 2. Variability of loci used in the 60 isolates tested[a]

Locus	No. of bp sequenced	No. of genotypes	No. (%) of variable sites	No. of parsimony-informative sites	No. of synonymous sites	No. of genotypes/variable base
CHS	280	8	13 (4.6)	11	13	0.61
Bt2	410	13	42 (10.24)	37	5	0.30
CAL	776	21	100 (12.8)	84	5	0.21

[a] Abbreviations: CHS, chitin synthase gene; Bt2, β-tubulin gene; CAL, calmodulin gene.

The number of nonsynonymous and synonymous mutations was calculated by the method of Nei and Gojobori (15) implemented in the DnaSP program (19). The most parsimonious trees were produced in PAUP* (phylogenetic analysis using parsimony and other methods), version 4.0b10 (23). One hundred heuristic searches were performed by random sequence addition and tree bisection-reconnection branch-swapping algorithms, collapsing zero-length branches, and saving all minimal-length trees (MulTrees) on different sets of data. Gaps were treated as missing data. Support for internal branches was assessed by a heuristic parsimony search of 500 bootstrapped sets of data. The combined data set was tested for incongruence with the partition homogeneity test as implemented in PAUP*. To test alternative phylogenetic relationships, the Kishino-Hasegawa maximum-likelihood ratio test (12) was performed as implemented in PAUP*.

Nucleotide sequence accession numbers. All of the sequences determined in this study were deposited in the EMBL database and assigned the accession numbers listed in Table 1.

RESULTS

With the primers used, we were able to amplify and sequence 280 bp, 410 bp, and 776 bp of the CHS, Bt2, and CAL loci, respectively, in 60 isolates of *S. schenckii* (Table 1). Of the 1,466 nucleotides sequenced, 1,311 were constant, 132 (9%) were parsimony informative, and 23 were variably parsimony noninformative. The lowest number was 11 in the CHS fragment, and the highest was 87 in the CAL fragment. The total numbers of nonsynonymous and synonymous changes were 0 and 23 (13 in CHS, 5 in Bt2, and 5 in CAL), respectively (Table 2). Sequences of the three genes were analyzed phylogenetically as separate and combined data sets.

In all of the trees obtained from the phylogenetic analysis of each locus, three main clades were shown. One comprised all of the isolates from Brazil (clade I), including also the only environmental isolate tested. Another clade (clade II) grouped the rest of the South American isolates and three more isolates, including the type strain. The third clade (clade III) grouped the European isolates, all from Spain, with the exception of two isolates of unknown origin.

The result of the partition homogeneity test showed that sequence data sets of the three loci were congruent ($P = 0.11$) and could therefore be combined. A total of 5,000 most parsimonious trees were produced by a heuristic search using the combined data set of 1,466 characters from the three loci. The trees had a consistency index of 0.925, a retention index of 0.991, and a homoplasy index of 0.074. A total of 29 different genotypes could be distinguished (Fig. 1). A total of 14 100% bootstrap-supported nodes were shown, representing six putative phylogenetic species. The clustering was similar to that observed in the particular trees of the different genes analyzed, especially with the CAL locus. The tree showed the same three main clades observed with the other loci, each of them receiving 100% bootstrap support.

The 18 isolates related to the long-lasting Brazilian outbreak of cat-transmitted sporotrichosis were located with the rest of the isolates from this country in clade I. They were isolated from 11 patients (Table 3). Four patients had more than one isolate. In two cases (P3 and P8), these belonged to a single genotype. Patients P4 and P9 were infected by two different genotypes each. The 18 isolates belonged to a total of six genotypes. Genotype G1 was the most common, infecting seven patients. Genotypes G1 and G2 were also present in other patients from Brazil not related to the outbreak.

Clade II was divided into two highly (100%) supported subclades (IIa and IIb). The Peruvian isolates, which were the most numerous after the Brazilian ones, were distributed into two different branches (IIa-1 and IIb-1), each with 100% bootstrap support. Subclade IIa was further divided into IIa-1, which included isolates from other countries (Bolivia, Colombia, and Argentina), and IIa-2, which had one isolate of unknown origin and the type strain. A similar distribution was observed in subclade IIb, in which the Peruvian branch (IIb-1) formed a phylogenetic group clearly separated from the other branch (IIb-2) comprising two isolates, one from South Africa and one from Argentina. This analysis showed the existence of a European clade (clade III). The latter clade was the most phylogenetically distant.

DISCUSSION

This report contains the results of a molecular phylogenetic analysis of the *S. schenckii* species complex inferred from DNA sequence data from three different loci. One of the most interesting results is the discovery that the isolates of *S. schenckii*, practically all of clinical origin, were grouped into six putative phylogenetic species. These cryptic species were further subdivided into a number of smaller groups that appear to be reproductively isolated in nature. This suggests not only that the existing *S. schenckii* populations are in the process of divergence but also that all of the resulting lineages are undergoing separation into distinct taxa. Our results were validated by the recent study of Neyra et al. (16), who analyzed the genetic diversity of the same Peruvian isolates that we tested, by using amplified fragment length polymorphism analysis and ITS2 sequencing. In that study, the isolates were grouped into two clades that were similar to those obtained in our phylogenetic trees.

Another interesting aspect of our analysis was the finding that each of the main groups exhibited a degree of geographical specificity, which agrees with previous reports (14, 16). The possible existence of different species within *S. schenckii* was already suggested by Wilhelm de Beer et al. (27) on the basis of the analysis of the sequences of the ITS regions of 11 clinical
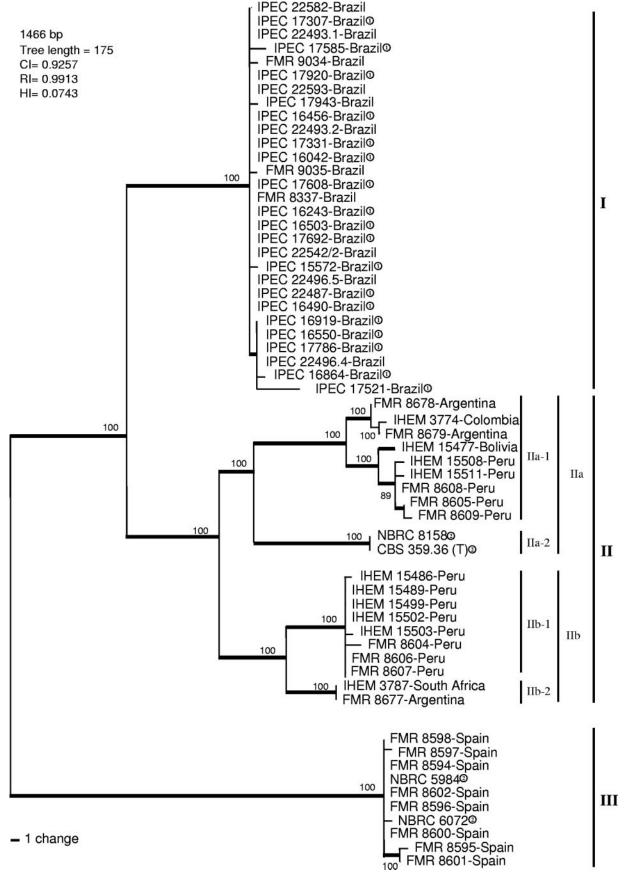
FIG. 1. One of the 5,000 most parsimonious trees obtained from heuristic searches based on an analysis produced from the combined data set. Bootstrap support values above 80% are indicated at the nodes. Branches present in the strict consensus tree are in bold. CI, consistency index; RI, retention index; HI, homoplasy index; T, type strain. Circled numeral 1, isolate from the Brazilian outbreak; circled numeral 2, origin not known.

TABLE 3. Genetic relationship between *S. schenckii* clinical isolates from the Brazilian cat-transmitted outbreak

Patient	Isolate[a]	Date	Genotype
P1	IPEC 16042	29/04/1999	G1
P2	IPEC 16243	11/06/1999	G1
P3	IPEC 16490	09/08/1999	G1
P3	IPEC 16503	12/08/1999	G1
P4	IPEC 16550	19/08/1999	G2
P4	IPEC 16864	04/11/1999	G3
P4	IPEC 16919	22/01/1999	G2
P5	IPEC 15572	19/11/1998	G4
P6	IPEC 16456	29/07/1999	G1
P7	IPEC 17307	16/03/2000	G1
P8	IPEC 17331	19/10/2000	G1
P8	IPEC 17608	01/06/2000	G1
P8	IPEC 17692	16/06/2000	G1
P8	IPEC 17920	17/08/2000	G1
P9	IPEC 17786	13/07/2000	G2
P9	IPEC 17521	04/05/2000	G5
P10	IPEC 17585	25/05/2000	G6
P11	IPEC 22487	30/01/2003	G1

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and environmental isolates from South Africa. Our results disagree with those of Ishizaki (10), who found, by using mitochondrial DNA analysis, that all of the clinical isolates studied (more than 500) belonged to the same species.

In our study, the combined analysis of the three loci revealed the presence of two major groups, one including the European isolates and the other the South American and South African isolates (Fig. 1). In the latter group, two clades were observed, one formed by the 29 Brazilian isolates and the second by the rest. These results agree with those of Neyra et al. (16), in whose amplified fragment length polymorphism study the European isolates, two from Belgium and one from Italy, were also the most distantly placed and clearly separated from the American isolates. In our study, one South African isolate was close to one South American isolate. This result is in agreement with that of Ishizaki et al. (11), who found, by using restriction fragment length polymorphism analysis of mitochondrial DNA, that the South American isolates studied were related to the South African isolates studied. The 24 mitochondrial DNA restriction fragment length polymorphism types described were classified into two large phylogenetic groups, one predominant in South America and Africa and the other predominant in Australia and Asia. The biogeographical pattern shown in all of these studies seems to correlate with the events associated with the formation of natural barriers created by the fragmentation of the ancient supercontinent Gondwana in the upper Cretaceous period through the Paleocene period over the last 100 million years. O'Donnell et al. proposed this to account for the radiating speciation of the *Gibberella fujikuroi* species complex (18).

Although the genetic separation is considerable among the three major monophyletic clades, i.e., the Spanish clade, the Brazilian clade, and the clade made up of the rest of the South American isolates, each of them shows a high level of clonality. Primitive populations were probably isolated by the separation of the continents, and the formation of natural barriers facilitated their speciation as they became adapted to hosts en-

demic to the different regions. However, although geographical separation of the main clades is clearly evident, the different genotypes present within them are not related to geography (16), which seems to indicate that there has been interbreeding within these isolated populations.

Our analysis revealed that six different genotypes were present in a sample of 18 isolates from the Brazilian outbreak of cat-transmitted sporotrichosis, which demonstrates that the infections are not caused by a single, highly virulent genotype, as could be thought. In the city of Rio de Janeiro and the surrounding areas, from 1987 to 1997, only 13 cases of human sporotrichosis were recorded, but from 1998 to 2004, 759 human cases were reported (21). It is difficult to explain this dramatic increase in *Sporothrix* infections in this region. The researchers who have reported all of these cases are unable to provide an explanation for this epidemic (21). However, it should be noted that the highest number of cases occurred in an area characterized by underprivileged socioeconomic conditions and precarious health services. The typical human patients were female, mainly housewives, which is normal if we consider that members of this group are those most frequently exposed to the fungus because they care for cats (22). Barros et al. (1) explained the wide dissemination of the disease with factors related to the behavior of cats which, although cohabiting with human beings, do not always stay in the house but also circulate in the neighborhood, often getting involved in fights with other animals and coming into contact with soil and plants.

In conclusion, *S. schenckii* appears to be a complex of species, some prevailing in certain geographical regions. An accurate knowledge of species limits could be of high medical interest, as they may show different clinical patterns and respond differently to therapy. For instance, the Brazilian isolates present a distinctive clinical picture with immune manifestations (erythema multiforme) (9), disseminated cutaneous lesions, and atypical forms (20).

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