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Rapid Identification of *Sporothrix* Species by T3B Fingerprinting

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This article describes PCR fingerprinting using the universal primer T3B to distinguish among species of the *Sporothrix* complex, *S. brasiliensis*, *S. globosa*, *S. mexicana*, and *S. schenckii*. This methodology generated distinct banding patterns, allowing the correct identification of all 35 clinical isolates at the species level, confirmed by partial calmodulin (CAL) gene sequence analyses. This methodology is simple, reliable, rapid, and cheap, making it an ideal routine identification system for clinical mycology laboratories.

Sporotrichosis is a globally distributed subcutaneous mycosis with areas of high endemicity (11, 21) that is caused by the dimorphic fungus *Sporothrix schenckii* (22). Sporotrichosis has been regarded as a job-related disease occurring as isolated cases or small outbreaks affecting people exposed to plants or soil (1, 6, 7). Rio de Janeiro State, Brazil, is a region of sporotrichosis hyperendemicity where several human and animal cases have been described since 1998 (5, 23).

The diagnosis of sporotrichosis is attained by clinical, epidemiological, and laboratorial data, including culture and analysis of phenotypic characteristics. The first description of PCR for sporotrichosis' diagnosis was reported in 2001 (9). A diagnostic nested PCR assay targeting the *S. schenckii* 18S rRNA gene was further evaluated and showed high sensitivity and specificity, indicating that PCR may be clinically useful for diagnosis (8).

S. schenckii was long considered a single taxon, although great genetic variation within this species has been described (10). By associating phenotypic and genotypic features, Marimon et al. (13) recognized three new species, *Sporothrix brasiliensis*, *Sporothrix globosa*, and *Sporothrix mexicana*, and proposed an identification key for these *Sporothrix* species. *S. globosa* has worldwide distribution (12, 18), whereas *S. brasiliensis* is apparently restricted to Brazil (13) and *S. mexicana* to Mexican environmental samples (13), although the latter was recently identified in Portugal (3). Additionally, these authors have proposed the promotion of *S. schenckii* var. *luriei* to the status of a new species, *Sporothrix luriei* (14). However, identification based only on phenotypic characteristics is often inconclusive due to phenotypic variability within these species. Therefore, new rapid and reliable identification strategies are necessary (19).

Analysis of tRNA intergenic spacers was first used to distinguish *Streptococcus* species (15). It has also been applied successfully for *Candida* identification (2, 16, 24). Here, we evaluate T3B PCR fingerprinting to differentiate clinical *Sporothrix* strains at the species level in comparison to analysis of partial calmodulin (CAL) gene sequences (19).

Thirty-five *Sporothrix* spp. isolates from the Fungal Culture Collection of IPEC/Fiocruz were included in this study approved by the Ethics Commission of the same institution. Among them, *S. brasiliensis* type strain CBS 120339 (IPEC16490), *S. globosa* IPEC27135 (18), *S. schenckii* IPEC29334 (IOC1226) (19), and *S. mexicana* (MUM11.02) (3) were used as controls. All 35 strains were previously phenotypically characterized (3, 19), and 15 isolates could not be identified (Table 1).

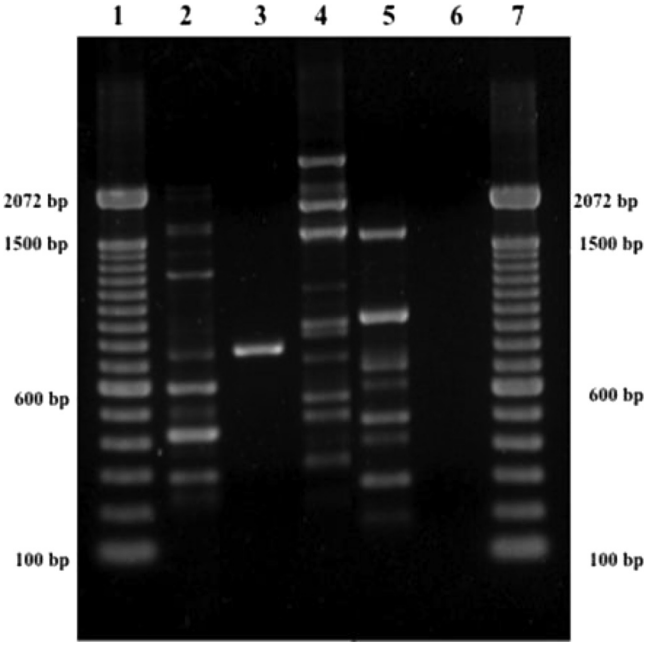

FIG 1 Representative T3B PCR fingerprinting profiles of the *Sporothrix* complex. (1 and 7) Molecular marker DNA ladder, 100 bp (Invitrogen). (2) *S. brasiliensis* (IPEC 16490). (3) *S. globosa* (IPEC 27135). (4) *S. mexicana* (MUM 11.02). (5) *S. schenckii* (IPEC27722). (6) Negative control.

Genomic DNA was extracted from the mycelial phase (25), and PCR was performed with the primer T3B (5′-AGGTCGCGGGT TCGAATCC-3′) (24). T3B PCR fingerprinting reproducibility was confirmed by repeating the assays at least 3 times under the same conditions at two different laboratories. The T3B profiles

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TABLE 1 Comparison of tools applied in the characterization of strains of the *Sporothrix* complex

Strain	Phenotypic identification[a]	Genotypic characterization		
		Final identification[b]	GenBank accession no.[c]	Reference[d]
IPEC16490	*S. brasiliensis*	*S. brasiliensis*	AM116899	19
IPEC27445-3	*S. brasiliensis*	*S. brasiliensis*	HQ426950	19
IPEC27052	*Sporothrix* sp.*	*S. brasiliensis*	HQ426941	19
IPEC27135	*Sporothrix* sp.*	*S. globosa*	GU456632	3
IPEC27387	*Sporothrix* sp.*	*S. brasiliensis*	HQ426948	19
IPEC34067	*Sporothrix* sp.*	*S. brasiliensis*	HQ426952	19
IPEC27372	*Sporothrix* sp.*	*S. brasiliensis*	HQ426947	19
IPEC25011	*S. brasiliensis*	*S. brasiliensis*	HQ426935	19
IPEC33605	*Sporothrix* sp.*	*S. brasiliensis*	HQ426957	19
IPEC27930	*Sporothrix* sp.*	*S. brasiliensis*	HQ426951	19
IPEC28772	*Sporothrix* sp.*	*S. brasiliensis*	HQ426955	19
IPEC28457	*S. brasiliensis*	*S. brasiliensis*	JN995607	This study
IPEC34007	*Sporothrix* sp.*	*S. brasiliensis*	HQ426959	This study
IPEC27177-2	*Sporothrix* sp.*	*S. brasiliensis*	HQ426944	19
IPEC27087	*S. brasiliensis*	*S. brasiliensis*	HQ426942	19
IPEC27288	*Sporothrix* sp.*	*S. brasiliensis*	HQ426945	19
IPEC27209	*Sporothrix* sp.*	*S. brasiliensis*	HQ426946	19
IPEC28604	*S. brasiliensis*	*S. brasiliensis*	HQ426953	19
IPEC26945	*Sporothrix* sp.*	*S. brasiliensis*	HQ426939	19
IPEC27130	*Sporothrix* sp.*	*S. brasiliensis*	HQ426943	19
IPEC25521	*Sporothrix* sp.*	*S. brasiliensis*	HQ426936	19
IPEC16919	*S. brasiliensis*	*S. brasiliensis*	HQ426930	19
IPEC18782A	*S. brasiliensis*	*S. brasiliensis*	HQ426933	This study
IPEC28329	*S. schenckii**	*S. brasiliensis*	JN995610	This study
IPEC27022	*S. brasiliensis*	*S. brasiliensis*	HQ426940	19
IPEC28487	*S. brasiliensis*	*S. brasiliensis*	HQ426928	19
IPEC28665	*S. brasiliensis*	*S. brasiliensis*	JN995606	This study
IPEC28790	*S. brasiliensis*	*S. brasiliensis*	HQ426956	19
IPEC29334	*S. schenckii*	*S. schenckii*	HQ426962	19
IPEC26961	*S. schenckii*	*S. schenckii*	JN995605	This study
IPEC27157-1	*S. schenckii*	*S. schenckii*	JN995604	This study
IPEC27100	*S. schenckii**	*S. brasiliensis*	JN995609	This study
IPEC27133	*S. schenckii**	*S. brasiliensis*	JN995608	This study
MUM 11.02	*S. mexicana* or *S. schenckii**	*S. mexicana*	JF970258	4
IPEC27722	*S. mexicana**	*S. schenckii*	HQ426961	19

[a] Performed according to reference 19. Asterisks indicate incorrect phenotypic identifications.
[b] Calmodulin sequencing and T3B concordant identification.
[c] Of strain used for characterization.
[d] Reference from which the partial gene calmodulin sequencing result was obtained.

were analyzed using the software Bionumerics 5.1 (Applied Maths BVBA, Saint-Martens-Latem, Belgium). Similarity coefficients were calculated using the Dice algorithm, and cluster analysis was performed by means of the unweighted-pair group method using average linkages (UPGMA). Sequencing of the CAL gene was performed as previously described (17) using the sequencing platform at Fiocruz, Brazil (20). Sequences were edited with Sequencer 4.6 (Genes Codes Corporation), aligned with MEGA 4.0.2 software, and compared with sequences available from NCBI GenBank by BLAST. Phylogenetic analyses were performed using MEGA with 1,000 bootstrap replicates (4) (http://www.megasoftware.net/).

T3B PCR fingerprinting of control strains showed profiles with DNA fragments ranging from 300 to 1,500 bp that allowed a clear distinction of *S. brasiliensis*, *S. globosa*, *S. mexicana*, and *S. schenckii* (Fig. 1). A dendrogram derived from analysis of the T3B profiles of all isolates splits *Sporothrix* strains into three groups, not the expected four, with IPEC27722, the only strain phenotypically characterized as *S. mexicana*, grouping into the *S. schenckii* cluster. Regardless of this discrepancy, the T3B profiling showed a high correspondence between clusters and *Sporothrix* species, with all isolates clustering with their respective control strain (Fig. 2). The T3B fingerprinting identification was confirmed by comparison with the CAL gene partial sequences obtained along with sequences from the NCBI database, AM398393.1 (*S. mexicana*), AM398382.1 (*Sporothrix albicans*), AM116908 (*S. globosa*), and AM117444.1 (*S. schenckii*). The phylogenetic tree of the CAL locus analyzed by neighbor joining revealed five distinct clades represented by the five species (Fig. 3). Overall, there was 100% agreement between T3B PCR fingerprinting and CAL locus sequencing on species identification. Comparing these results with phenotypic analysis, 14 of the 15 isolates with inconclusive phenotypes

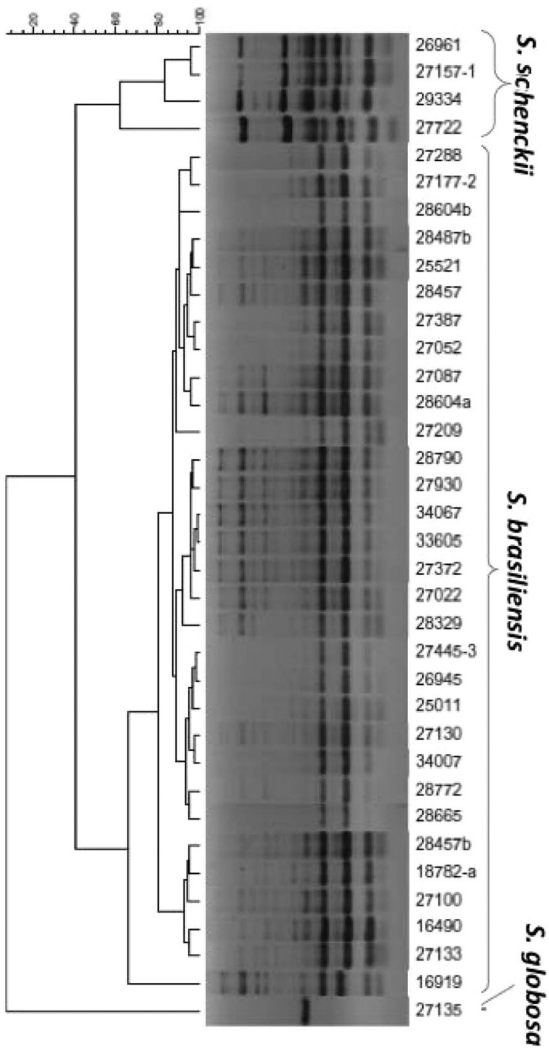

FIG 2 Dendrogram showing the degrees of similarity of T3B fingerprinting profiles among the *Sporothrix* isolates by using the Dice coefficient and the UPGMA cluster method. The cophenetic correlation coefficient (0.97) indicates a very good fit for this analysis.

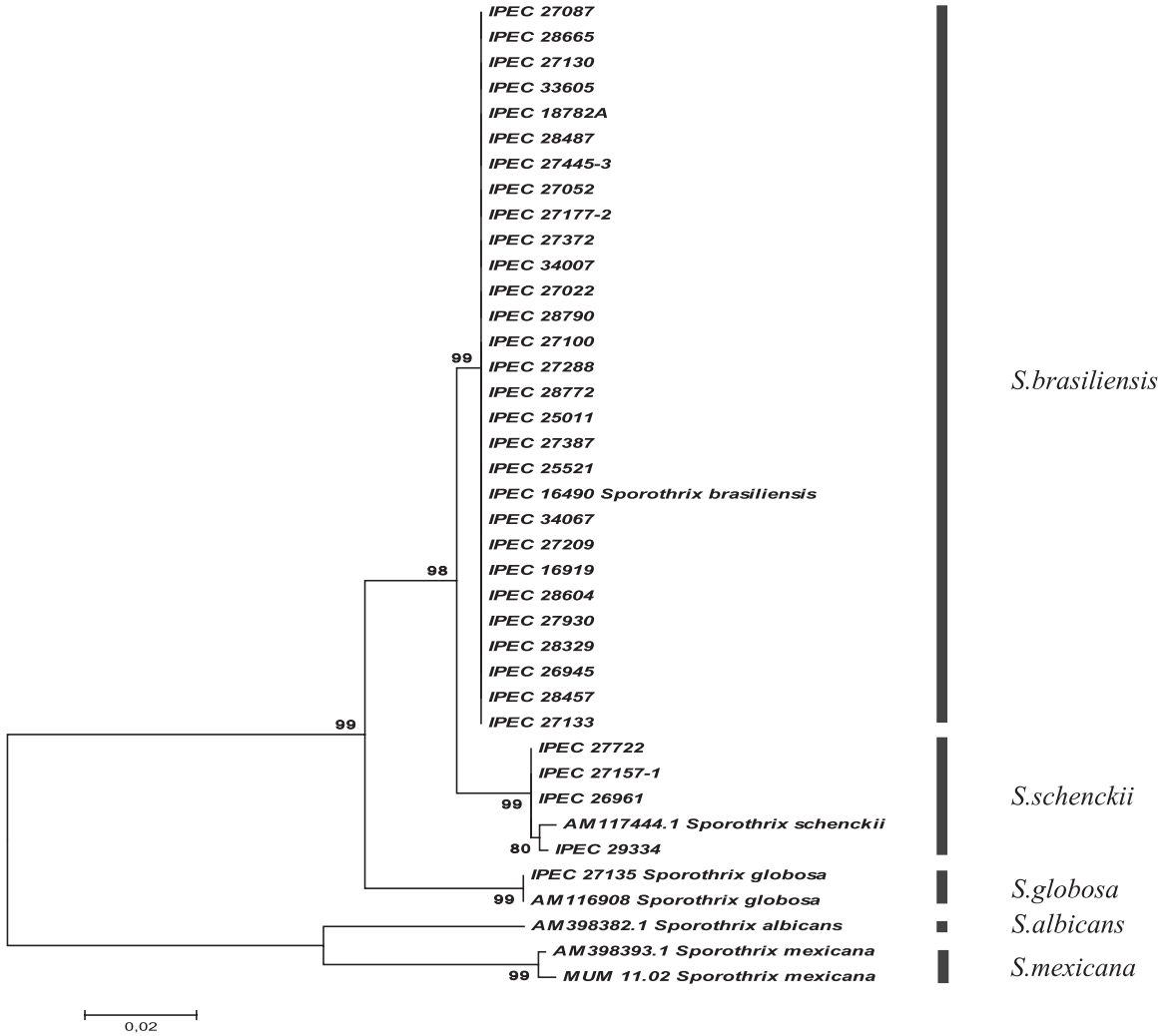

FIG 3 Consensus tree of *Sporothrix* based on partial calmodulin (CAL) gene sequences of 35 strains and the NCBI public GenBank sequences AM398393.1 (*S. mexicana*), AM398382.1 (*S. albicans*), and AM117444.1 (*S. schenckii*) that was constructed with MEGA version 4.0.2 and 1,000 bootstrap replicates.

grouped within the *S. brasiliensis* cluster and IPEC27135 grouped within the *S. globosa* cluster. The genotypic analyses also allowed the correction of phenotypic misidentifications, such as for the isolate IPEC27722, which was phenotypically characterized as *S. mexicana* but grouped within the *S. schenckii* cluster, as well as for the isolates IPEC27133, IPEC27100, and IPEC28329, which identified as *S. schenckii* but clustered with *S. brasiliensis*.

Identification of the *Sporothrix* species complex has been based on a polyphasic approach using a combination of phenotypic methodologies and sequencing (13, 14, 18, 19). The proposed identification key based on phenotypic tests (13) was reported as easy and reliable for species differentiation without the need of molecular techniques. However, the results are often inconclusive or ambiguous, and some species are too closely related to show phenotypic differences. This work describes the assessment of DNA polymorphism within the *Sporothrix* complex by genomic DNA amplification with the single nonspecific primer T3B. The T3B PCR profiles were highly informative and generated clearly distinct banding patterns for each species, allowing their differentiation. *S. brasiliensis* strains showed bands sharing similarity higher than 80%, but intraspecies variation was observed. This variation was previously demonstrated for *Candida* (2), and band-sharing values observed are within the variation range (70 to 85%) expected for strains within the same species (16).

Comparison of the inconclusive phenotypic identification of isolates with the results obtained by T3B PCR fingerprinting demonstrated the sensitivity of the latter, since species of those strains could all be determined. This proposed identification technique is simple, reliable, rapid, and cheaper and requires less technical expertise than sequencing. The generated, computer-scanned PCR profiles can form the basis of a database which can be used for future identifications of atypical or unidentifiable *Sporothrix* isolates. Furthermore, these advantages are especially valuable in a laboratory with limited facilities, making it an ideal identification methodology for clinical mycology laboratories.

Nucleotide sequence accession numbers. All sequences were deposited in the GenBank database under accession numbers GU456632, HQ426928 to HQ426962, and JN995604 to JN995610.

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Automated sequencing was done using the genomic platform/DNA sequencing platform at Fundação Oswaldo Cruz—PDTIS/FIOCRUZ (RPT01A), Brazil.

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