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# Evaluation of T3B fingerprinting for identification of clinical and environmental *Sporothrix* species

Manoel Marques Evangelista Oliveira[1,*], Ricardo Franco-Duarte[2], Orazio Romeo[3], Célia Pais[2], Giuseppe Criseo[3], Paula Sampaio[2] and Rosely Maria Zancope-Oliveira[1]

[1]Laboratório de Micologia, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, RJ 21045-900, Brazil, [2]Molecular and Environmental Biology Centre (CBMA), Universidade do Minho, 4710-057, Braga, Portugal and [3]Department of Environmental and Biological Sciences - University of Messina, 98166, Italy

*Corresponding author: Setor de Imunodiagnóstico do Laboratório de Micologia do Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz. Av. Brasil 4365, Manguinhos, Rio de Janeiro, RJ 21045-900, Brazil. Tel: +(55-21) 3865-9640; Fax: +(55-21) 3865-9557;
E-mail: manoel.marques@ini.fiocruz.br

**One sentence summary:** In our study are described for first time the application of the PCR fingerprinting to distinguish all species, clinical and environmental, of an important fungic complex, *Sporothrix* spp.

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## ABSTRACT

In this study, PCR fingerprinting using the universal primer T3B was applied to distinguish among clinical and environmental species of the *Sporothrix* complex, *Sporothrix brasiliensis*, *S. globosa*, *S. mexicana*, *S. pallida*, *S. luriei* and *S. schenckii* sensu stricto. The T3B fingerprinting generated clearly distinct banding patterns, allowing the correct identification of all 43 clinical and environmental isolates at the species level, what was confirmed by partial calmodulin gene sequence analyses. This technique is reproducible and provides the identification of all species of the *Sporothrix* complex with sufficient accuracy to be applied in clinical mycology laboratories as well as in epidemiological studies in order to obtain a better understanding of the epidemiology of sporotrichosis.

**Key words:** *Sporothrix* species complex; molecular identification; sporotrichosis

## INTRODUCTION

Sporotrichosis is a chronic, granulomatous subcutaneous mycosis caused by pathogenic species in the *Sporothrix schenckii* complex. This infection is globally distributed, being Latin America, South Africa, India, China and Japan areas of high endemicity (Lopez-Romero *et al.* 2011; Queiroz-Telles *et al.* 2011; Song *et al.* 2013). Sporotrichosis occurs mainly through traumatic inoculation of fungal propagules into the skin by contaminated material, such as soil, plant thorns or splinters, being regarded as a job-related disease occurring in the form of isolated cases or small outbreaks affecting people exposed to plants or organic matter rich soil (Cooper, Dixon and Salkin 1992; Hajjeh *et al.* 1997; Zancopé-Oliveira *et al.* 2011). Sporotrichosis affects humans and animals, and its zoonotic potential has been well exemplified in outbreaks in Brazil due to animal scratches and bites (Schubach, Barros and Wanke 2008; Zancopé-Oliveira *et al.* 2011). Rio de Janeiro in Brazil has been reported as a hyperendemic region since, from 1997 to 2007, 1848 cases of human sporotrichosis occurred in that state (Schubach, Barros and Wanke 2008; Silva *et al.* 2012). Curiously, 83.4% of the human infections

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**Table 1.** Polyphasic taxonomy in characterization of strains of the *Sporothrix* complex and comparison with tool of T3B fingerprinting.

| Strain | Final identification[a] | Source | Genbank n° | References[b] |
|---|---|---|---|---|
| IPEC16490 | *S. brasiliensis* | Clinical | AM116899 | Oliveira *et al.* (2011) |
| IPEC27445-3 | *S. brasiliensis* | Clinical | HQ426950 | Oliveira *et al.* (2012) |
| IPEC27052 | *S. brasiliensis* | Clinical | HQ426941 | Oliveira *et al.* (2012) |
| IPEC27135 | *S. globosa* | Clinical | GU456632 | Oliveira *et al.* (2010) |
| INSA378027 | *S. globosa* | Clinical | KF437620 | Oliveira *et al.* (2014) |
| IPEC27387 | *S. brasiliensis* | Clinical | HQ426948 | Oliveira *et al.* (2011) |
| IPEC34067 | *S. brasiliensis* | Clinical | HQ426952 | Oliveira *et al.* (2011) |
| IPEC27372 | *S. brasiliensis* | Clinical | HQ426947 | Oliveira *et al.* (2011) |
| IPEC25011 | *S. brasiliensis* | Clinical | HQ426935 | Oliveira *et al.* (2011) |
| IPEC33605 | *S. brasiliensis* | Clinical | HQ426957 | Oliveira *et al.* (2011) |
| IPEC27930 | *S. brasiliensis* | Clinical | HQ426951 | Oliveira *et al.* (2011) |
| IPEC28772 | *S. brasiliensis* | Clinical | HQ426955 | Oliveira *et al.* (2011) |
| IPEC28457 | *S. brasiliensis* | Clinical | JN995607 | Oliveira *et al.* (2012) |
| IPEC34007 | *S. brasiliensis* | Clinical | HQ426959 | Oliveira *et al.* (2012) |
| IPEC27177-2 | *S. brasiliensis* | Clinical | HQ426944 | Oliveira *et al.* (2011) |
| IPEC27087 | *S. brasiliensis* | Clinical | HQ426942 | Oliveira *et al.* (2011) |
| IPEC27288 | *S. brasiliensis* | Clinical | HQ426945 | Oliveira *et al.* (2011) |
| IPEC27209 | *S. brasiliensis* | Clinical | HQ426946 | Oliveira *et al.* (2011) |
| IPEC28604 | *S. brasiliensis* | Clinical | HQ426953 | Oliveira *et al.* (2011) |
| IPEC26945 | *S. brasiliensis* | Clinical | HQ426939 | Oliveira *et al.* (2011) |
| IPEC27130 | *S. brasiliensis* | Clinical | HQ426943 | Oliveira *et al.* (2011) |
| IPEC25521 | *S. brasiliensis* | Clinical | HQ426936 | Oliveira *et al.* (2011) |
| IPEC16919 | *S. brasiliensis* | Clinical | HQ426930 | Oliveira *et al.* (2011) |
| IPEC18782A | *S. brasiliensis* | Clinical | HQ426933 | Oliveira *et al.* (2012) |
| IPEC28329 | *S. brasiliensis* | Clinical | JN995610 | Oliveira *et al.* (2012) |
| IPEC27022 | *S. brasiliensis* | Clinical | HQ426940 | Oliveira *et al.* (2011) |
| IPEC28487 | *S. brasiliensis* | Clinical | HQ426928 | Oliveira *et al.* (2011) |
| IPEC27375 | *S. brasiliensis* | Clinical | JN995606 | Oliveira *et al.* (2012) |
| IPEC28790 | *S. brasiliensis* | Clinical | HQ426956 | Oliveira *et al.* (2011) |
| IPEC29334 | *S. schenckii* | Clinical | HQ426962 | Oliveira *et al.* (2011) |
| IPEC26961 | *S. schenckii* | Clinical | JN995605 | Oliveira *et al.* (2012) |
| IPEC27157-1 | *S. schenckii* | Clinical | JN995604 | Oliveira *et al.* (2012) |
| IPEC27100 | *S. brasiliensis* | Clinical | JN995609 | Oliveira *et al.* (2012) |
| IPEC27133 | *S. brasiliensis* | Clinical | JN995608 | Oliveira *et al.* (2012) |
| MUM 11.02 | *S. mexicana* | Clinical | JF970258 | Dias *et al.* (2011) |
| CBS937.72 | *S. luriei* | Clinical | AM747302 | Marimon *et al.* (2008a) |
| BG6 | *S. pallida* | Environmental | HQ692915 | Romeo, Scordino and Criseo (2011) |
| BG | *S. pallida* | Environmental | KJ472127 | Romeo, Scordino and Criseo (2011) |
| BG2 | *S. pallida* | Environmental | KJ472128 | Romeo, Scordino and Criseo (2011) |
| SAM1 | *S. pallida* | Environmental | KJ472130 | Romeo, Scordino and Criseo (2011) |
| SPA8 | *S. pallida* | Environmental | HQ686039 | Romeo, Scordino and Criseo (2011) |
| SPA2 | *S. pallida* | Environmental | KJ472129 | Romeo, Scordino and Criseo (2011) |
| IPEC27722 | *S. schenckii* | Clinical | HQ426961 | Oliveira *et al.* (2011) |

[a]calmodulin sequencing and T3B identification concordant identification.
[b]Reference of partial gene calmodulin sequencing.

were associated with prior contact with infected cats (Schubach, Barros and Wanke 2008). This route of infection contrasts markedly with other sporotrichosis reports which have been mainly associated with infection via a plant source, rather than by domestic cats infected with *S. schenckii* (Hay and Morris-Jones 2008; Freitas *et al.* 2010). Confirming the worldwide distribution of the sporotrichosis, a large series of cases have been reported in Jilin province, Northeast China, demonstrating an endemic situation, with epidemiological and clinical characteristics similar to those of previous Chinese reports, but different from those in other countries, as for example in Rio de Janeiro, Brazil, where the endemia demonstrated zoonotic transmission (Zancopé-Oliveira *et al.* 2011; Song *et al.* 2013).

Until 2007, *S. schenckii* was considered a single taxon, although Liu *et al.* (2003) had previously reported the existence of high genetic variation within this species. Nowadays, it is recognized as *S. schenckii* complex comprising *S. brasiliensis*, *S. globosa*, *S. mexicana* and *S. luriei* (Marimon *et al.* 2007; Marimon *et al.* 2008a). Although geographic limitations are not precise, epidemiological data indicate that *S. schenckii* sensu stricto is found predominantly on the American, Asian and African continents; *S. globosa* has a worldwide distribution and it is found with high frequency in Europe and Asia (Madrid *et al.* 2009; Oliveira *et al.* 2010, 2014; Yu *et al.* 2013). *Sporothrix brasiliensis* is apparently restricted to Brazil (Marimon *et al.* 2007; Oliveira *et al.* 2011) while *S. mexicana* seems to be mainly associated with Mexican environmental samples (Marimon *et al.* 2007), although it has also been, recently, identified in Portugal (Dias *et al.* 2011) and in Brazil (Rodrigues, de Hoog and de Camargo 2013). *Sporothrix luriei* is a very rare pathogen, reported on four human sporotrichosis cases, but isolated only from one case in Africa (Marimon *et al.* 2008a).

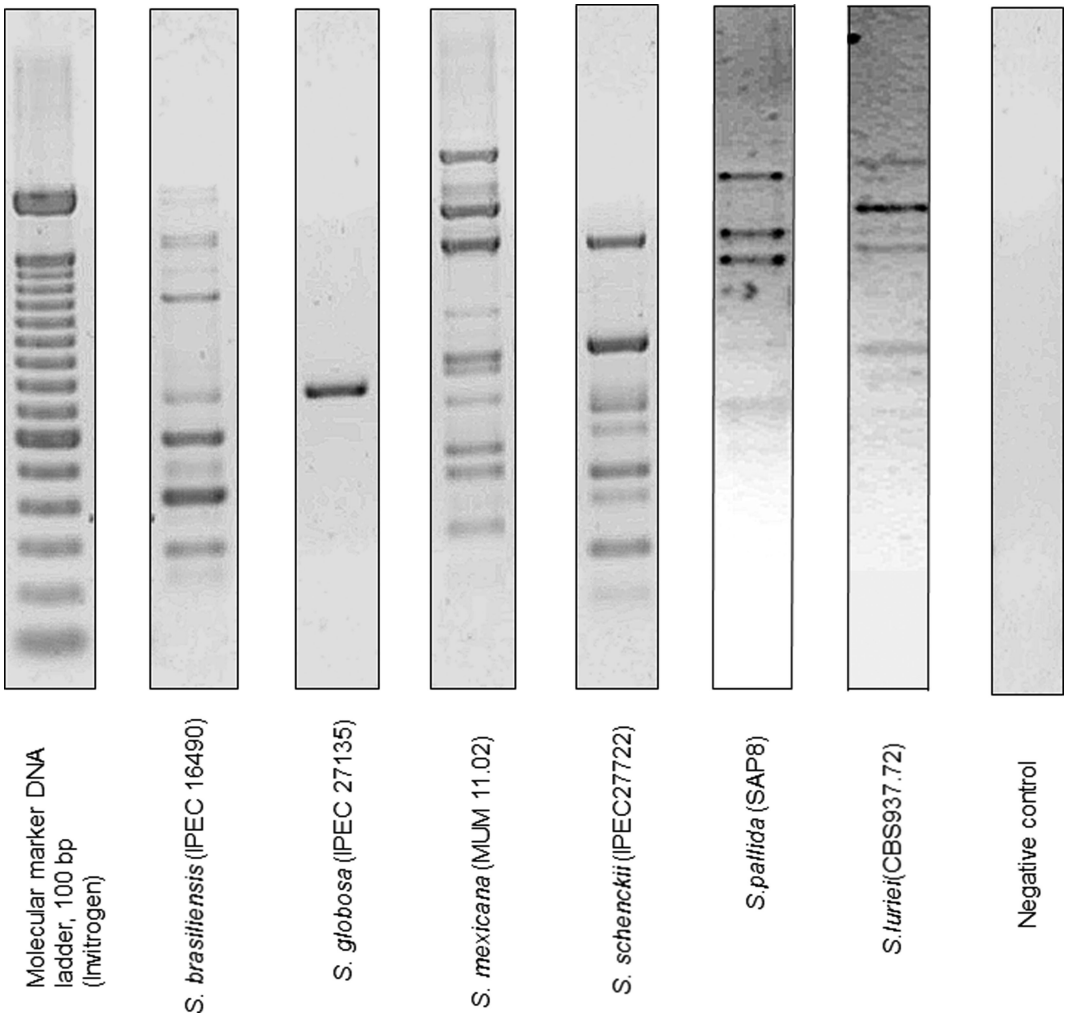


**Figure 1.** Representative PCR fingerprinting profiles obtained with primer T3B for *Sporothrix* the species. Lanes 1 and 8 (1) molecular marker DNA ladder 100 base pair; (2) *S. globosa* (IPEC 27135); (3) *S. brasiliensis* (IPEC 164904); (4) *S. mexicana* (MUM 11.02); (5) *S. schenckii* (IPEC27722); (6) *S. pallida* (SPA8); (7) *S. luriei* (CBS 937.72); (8) Negative control.

Phylogenetic analysis based on rDNA and the $\beta$-tubulin sequence regions from *S. albicans*, *S. pallida* and *S. nivea* revealed a high genetic similarity, and it was proposed to consider them as *S. pallida* (de Meyer *et al.* 2008). Until 2012, the species *S. pallida* was considered as environmental species, but a recent clinical report described its involvement in a case of keratitis in the cornea of a transplant recipient (Morrison *et al.* 2013).

Currently, medically relevant *Sporothrix* spp. in the *S. schenckii* complex are *S. brasiliensis*, *S. schenckii* s. str., *S. globosa* and *S. luriei*, while *S. mexicana* and *S. pallida* are phylogenetically more remote and, therefore, considered apart from the clinical group (Zhou, Feng and de Hoog 2014). Recent studies showed that the different *Sporothrix* spp. differ in virulence and drug resistance (Romeo and Criseo 2013). *Sporothrix brasiliensis* and *S. schenckii* were shown to be the most virulent species, contrasting with *S. globosa* and *S. mexicana* that showed little or no virulence in a murine model of disseminated infection (Arrillaga-Moncrieff *et al.* 2009). Curiously, *S. brasiliensis* seems to be the most susceptible species to several antifungal agents, while *S. mexicana* has been reported as the species most resistant showing only a relatively low MIC (0.5 g ml$^{-1}$) for terbinafine (Marimon *et al.* 2008b). Thus, once a culture is obtained, the identification to species level is mandatory because antifungal therapy can vary according to the species.

The diagnosis of sporotrichosis is classically attained by correlation of clinical, epidemiological and laboratorial data, including culture and analysis of phenotypic characteristics. An identification key for the *Sporothrix* species complex has been proposed which included conidial morphology and auxonogram analysis, using raffinose and sucrose as carbon sources (Marimon *et al.* 2007). However, identification based only on this phenotypic key is often inconclusive, due to phenotypic variability within the species (Oliveira *et al.* 2011; Rodrigues, de Hoog and de Camargo 2013; Zhou, Feng and de Hoog 2014). A variety of polymerase chain reaction (PCR)-based assays using different targets have been developed to identify *S. schenckii* but only few studies developed methodologies to distinguish more than *S. schenckii* from the *Sporothrix* spp. complex (Kanbe *et al.* 2005; Oliveira *et al.* 2012). We recently described a PCR fingerprinting using the universal primer T3B to distinguish among human pathogenic species of the *Sporothrix* complex, *S. brasiliensis*, *S. globosa*, *S. mexicana* and *S. schenckii* (Oliveira *et al.* 2012). In addition, a PCR-RFLP using with target the calmodulin gene digested with the restriction enzyme *Hha*I was reported, with five different electrophoretic patterns representing the isolates

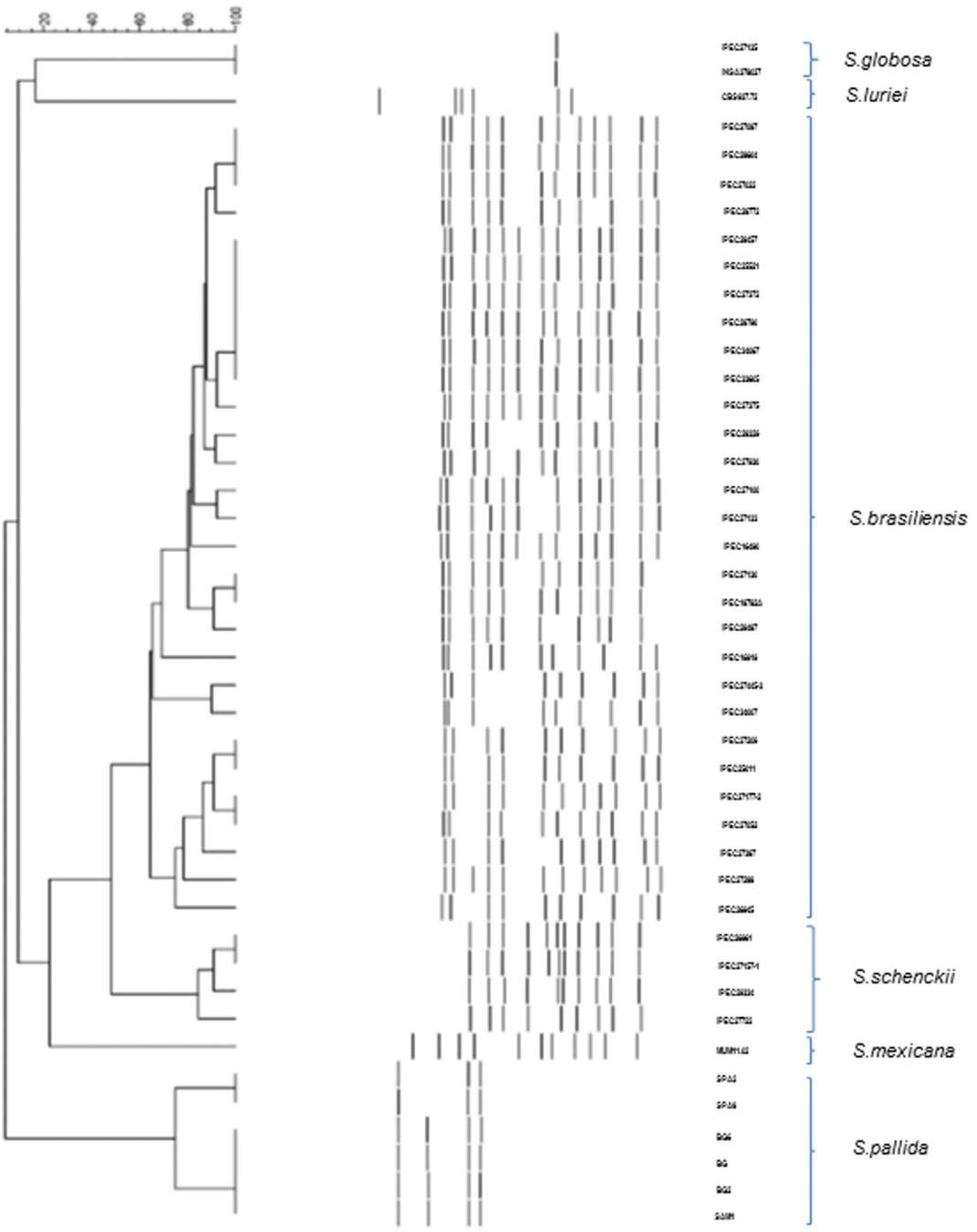


**Figure 2.** Dendrogram showing the degree of similarity of T3B fingerprinting profiles among the *Sporothrix* isolates by using the Dice coefficient and UPGMA cluster method. Cophenetic correlation coefficient (0.97) indicates a very good fit for this analysis.

of *Sporothrix* species: *S. brasiliensis*, *S. schenckii* sensu stricto, *S. globosa* and *S. luriei*. However, this PCR-RFLP protocol also not permitted identification of all isolates included in this complex (Rodrigues, de Hoog and de Camargo 2014). Here, we evaluate T3B PCR fingerprinting to differentiate environmental *Sporothrix* strains at the species level in comparison to analysis of partial calmodulin (CAL) gene sequences (Oliveira *et al.* 2010) and compared the obtained patterns with those previously identified in clinical *Sporothrix* isolates.

A total of 43 *Sporothrix* spp. isolates (Table 1), including the controls *S. brasiliensis* type strain CBS 120339 (IPEC16490) (Marimon *et al.* 2007), *S. globosa* IPEC27135 (Oliveira *et al.* 2010), *S. schenckii* s.str. IPEC29334 (IOC1226) (Oliveira *et al.* 2011), *S. mexicana* (MUM11.02) (Dias *et al.* 2011), *S. luriei* CBS937.72 (Marimon *et al.* 2008a) and *S. pallida* SPA8 (Romeo, Scordino and Criseo 2011) were used in this study. All strains were previously phenotypically and genotypically characterized at the species level (Table 1).

Genomic DNA was extracted from the mycelial phase, and PCR was performed with the primer T3B (5′-AGG TCG CGG GTT CGA ATCC-3′) according to Oliveira *et al.* (2012). The reproducibility of the method was confirmed by repeating the T3B PCR fingerprinting assays at least three times under the same conditions and in three different laboratories in Brazil,

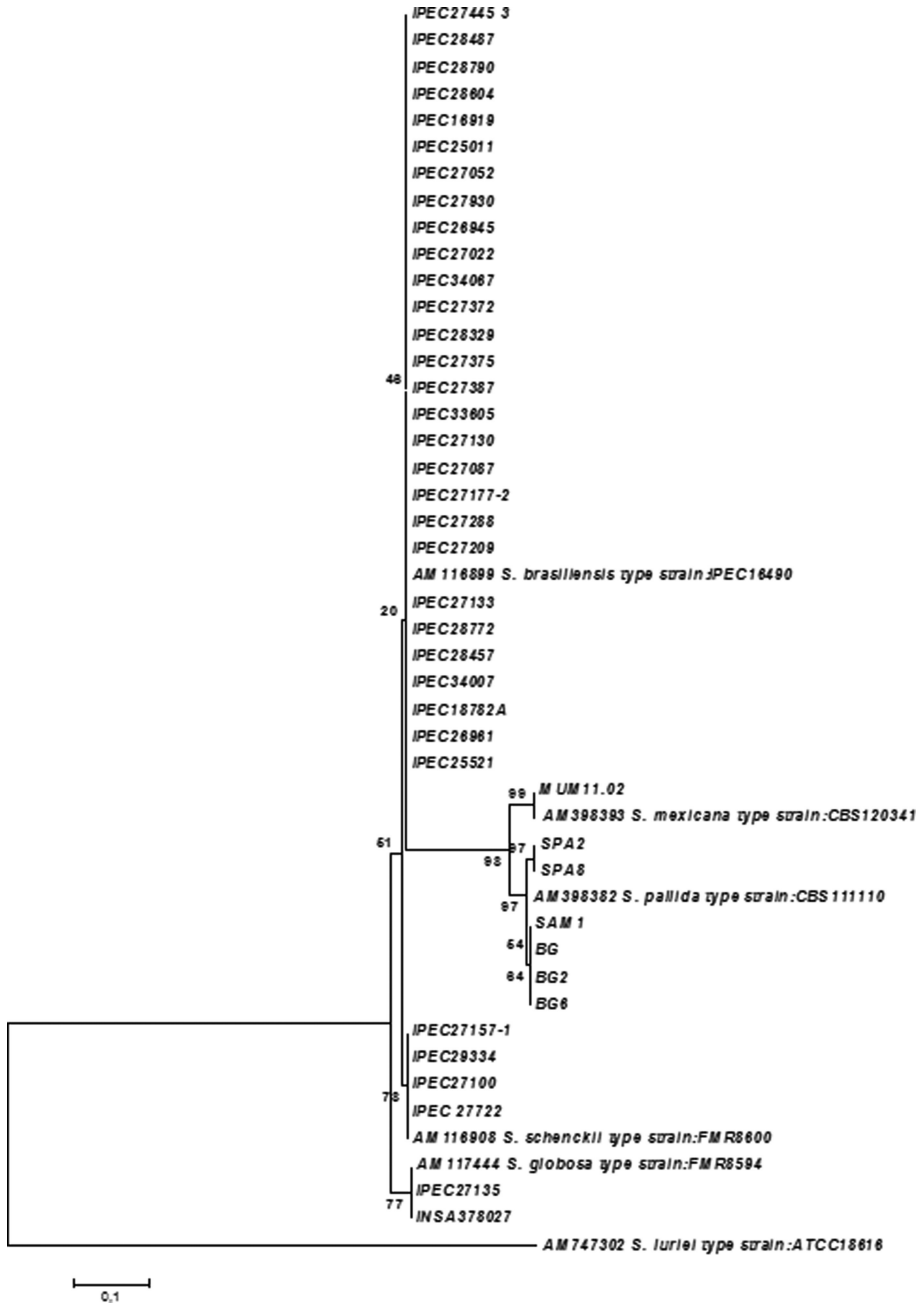


**Figure 3.** Neighbor-joining phylogram of the partial CAL gene obtained of all isolates of the study and *S. mexicana*, *S. pallida*, *S. brasiliensis*, *S. schenckii*, *S. luriei* and *S. globosa* reference strains constructed with MEGA version 4.0.2. Bootstrap values after 1000 replicates are presented in the branch node.

Portugal and Italy. The T3B fingerprinting profiles obtained were analyzed with Bionumerics (version 5.1; Applied Maths BVBA, Sint-Martens-Latem, Belgium). Similarity coefficients were calculated using the Dice algorithm and cluster analysis was performed by means of the unweighted paired group method using arithmetic averages (UPGMA). Partial calmodulin-encoding gene (CAL) sequences were obtained from previous studies (Table 1), edited with the Sequencer ver. 4.6 software package (Genes Codes Corporation, USA), and aligned with MEGA version 4.0.2 software (http://www.megasoftware.net/). Phylogenetic analyses were performed by using MEGA software with bootstrap analysis using 1000 replicates (Felsenstein 1985). All sequences were deposited in the GenBank database under accession numbers GU456632, HQ426928–HQ426962, JN995604–JN995610 and KJ472127–KJ472130.

The T3B PCR fingerprinting of *Sporothrix* spp. control strains showed profiles with DNA fragments ranging in size from 300 to 2800 bp, allowing the clear distinction of the strains from *S. brasiliensis*, *S. globosa*, *S. mexicana*, *S. schenckii*, *S. pallida* and *S. luriei* (Fig. 1). To confirm the taxonomic resolution of T3B amplification, the profiles of all isolates were analyzed. Although intraspecies T3B profiles were not 100% similar, a band sharing similarity higher than 80% was observed for *S. brasiliensis* strains and for *S. pallida* isolates was higher than 90%. The band sharing values observed in this study are within the range of variation (70–85%) considered for strains within the same

species (Meyer, Maszewska and Sorrell 2001; de Oliveira *et al.* 2012). The inter-species variation was sufficient to clearly differentiate all species and to group all isolates accordingly. This fingerprinting variation was also demonstrated previously by Oliveira *et al.* (2012) for *Sporothrix* strains and for *Candida* spp. (Correia *et al.* 2004). A dendrogram derived from analysis of the T3B profiles of all isolates splits the *Sporothrix* strains into six groups, showing a high correspondence between clusters and *Sporothrix* species, with all isolates clustering with their respective control strain (Fig. 2). The CAL gene partial sequences of the studied isolates along with sequences from the NCBI database, AM398393.1 (*S. mexicana*), AM398382.1 (*S. pallida*), AM117444.1 (*S. schenckii*), AM116899 (*S. brasiliensis*), AM116908 (*S. globosa*) and AM747302 (*S. luriei*) were analyzed. The phylogenetic tree of the CAL locus analyzed by neighbor joining revealed six distinct clades representing the six species (Fig. 3).

Analyses of the results obtained with T3B fingerprinting identification showed 100% concordance with results from partial sequencing of the CAL gene, confirming the accuracy of T3B fingerprinting.

The identification of the *Sporothrix* species complex was based on a polyphasic approach using a combination of phenotypic methodologies and sequencing (Marimon *et al.* 2007; de Oliveira *et al.* 2010, 2011; Dias *et al.* 2011), but phenotypic tests proposed by Marimon *et al.* (2007) are often inconclusive or ambiguous, and some species are too closely related to show clear-cut differences (Oliveira *et al.* 2011; Rodrigues, de Hoog and de Camargo 2013; Zhou, Feng and de Hoog 2014). In this study, we showed for the first time that T3B fingerprinting has the accuracy to identify all species of the *Sporothrix* complex. The inclusion of the *S. pallida* is very important because, although initially described as environmental species (Marimon *et al.* 2007; de Meyer *et al.* 2008; Romeo, Scordino and Criseo 2011), recently it was reported as etiologic agent of human sporotrichosis (Morrison *et al.* 2013).

The T3B PCR fingerprinting technique is reproducible, reliable, rapid and less expensive, requires less technical expertise than sequencing and has a 100% agreement on species identification as the sequencing of the CAL locus. Thus, T3B fingerprinting could represent a useful tool in epidemiological studies in order to obtain a better understanding of the role of these new *Sporothrix* species in causing human infection.

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***Conflict of interest statement.*** None declared.

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