Mycopathologia (2011) 172:179–186
DOI 10.1007/s11046-011-9420-z

New Insight into Molecular Phylogeny and Epidemiology of *Sporothrix schenckii* Species Complex Based on Calmodulin-Encoding Gene Analysis of Italian Isolates

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Received: 14 December 2010 / Accepted: 22 March 2011 / Published online: 2 April 2011

Abstract In this study, we investigated phylogenetic relationships among Italian *Sporothrix schenckii* isolates, by comparing their partial calmodulin sequences. In this analysis, we used 26 environmental strains of *S. schenckii*, plus two autochthonous clinical isolates. The results showed that our clinical strains grouped with *S. schenckii* sensu stricto isolates, whereas all 26 environmental isolates co-clustered with *Sporothrix albicans* (now regarded as a synonym of *Sporothrix pallida*), a non-pathogenic species closely related to *S. schenckii*. Furthermore, the group of environmental strains was found to be quite heterogeneous and further subdivided into two subgroups. The data reported here also showed that molecular methods, for specific identification of *S. schenckii,* developed before the description of its closely related species should be used with caution because of the possibility of false positive results, which could lead to inappropriate antifungal therapy. This study improves our understanding of the distribution of these new closely related *Sporothrix* species which also showed significant differences in antifungal susceptibilities.

Keywords *Sporothrix* complex · *Sporothrix schenckii* · *Sporothrix albicans* · Sporotrichosis · Calmodulin gene · Chitin synthase gene

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Introduction

Sporothrix schenckii is a pathogenic dimorphic fungus that causes sporotrichosis, a cutaneous lymphatic or systemic mycosis particularly frequent in certain geographical areas such as Mexico, Brazil, Peru, and India [1–3]. However, infections due to *S. schenckii* have also been reported from other parts of the world, including Europe, where sporotrichosis is considered a rare disease [4–6]. Nevertheless, in recent years, several clinical autochthonous cases have been described in patients and animals that live in European countries [7–12], showing that this pathogenic fungus is more widespread than is now believed. In fact, a high prevalence of *S. schenckii* has been recently found in some kind of commercial soils which might represent an important vehicle for introducing the fungus in nature [13]. Therefore, it is not clear why, despite the occurrence of *S. schenckii* in environmental and commercial samples, sporotrichosis has a low incidence in Italy. Different

assumptions were made in this regards, and one possible explanation could be the existence of cryptic species with reduced or absent pathogenicity [13]. This is in accordance with previous data obtained from phenotypic, virulence, and molecular studies which have suggested that more than one species could exist within *S. schenckii* [14–18]. In addition, more recently, Marimon et al. [18] have further supported this idea by examining three protein-coding loci, and on the bases of such studies, *S. schenckii* can now be recognized as a species complex comprising at least six sibling species: *Sporothrix albicans*, *Sporothrix brasiliensis*, *Sporothrix globosa*, *Sporothrix luriei*, *Sporothrix mexicana,* and *S. schenckii* [19, 20]. Furthermore, subsequent phylogenetic analysis based on sequence data of ribosomal DNA and β-tubulin regions from *Sporothrix pallida, Sporothrix nivea,* and *S. albicans* revealed a remarkably high genetic similarity, and therefore the last two species were synonymized with *S. pallida*, the first of the three species to be described [21]. However, apart from *S. schenckii* sensu stricto, only *S. brasiliensis*, *S. globosa* and *S. luriei* are associated with human infections, whereas *S. mexicana* and *S. pallida* have only been isolated from environmental samples even if *S. pallida* has been sometimes found as part of the intestinal flora of some insects [22]. It also is assumed that these two species of *Sporothrix* are not-infective to humans and other animals, and a recent experimental in vivo study has further confirmed that these fungi are non-pathogenic in a murine model of infection [23].

Molecular phylogeny, based on partial calmodulin sequence data, has significantly improved resolution among closely related *Sporothrix* species establishing six well-defined groups [19, 20]. Therefore, in this study, we amplify and sequence the calmodulin-encoding gene to evaluate the phylogenetic group to which the Italian *Sporothrix* isolates belong to. This is important for epidemiological investigations because it can improve our understanding of the distribution of these newly described *Sporothrix* species that have also showed significant differences in antifungal susceptibilities in vitro [24]. In fact, Marimon et al. [24] reported that the terbinafine was the most active antifungal agent against all *Sporothrix* species complex, whereas all others tested antimicotic drugs showed very poor activity against *S. pallida*, *S. globosa*, and *S. mexicana*.

Materials and Methods

Twenty-six environmental strains of *S. schenckii,* plus two clinical isolates, were examined in this study (Table 1). All strains were isolated in Italy and have already been well characterized, phenotypically and genotypically, in our previous study [13]. Nevertheless, however, their identity was further confirmed before use by standard phenotypic and molecular methods. *S. schenckii* ATCC 10268, *S. schenckii* KMU 975, *S. luriei* KMU 2787, *S. mexicana* FMR 9108, *S. brasiliensis* SS74, *S. globosa* SS49, and *S. albicans* CBS 302.73 were also included and used as reference strains.

For demonstration of thermal dimorphism, one-week-old fungal cultures of the mycelial form on Sabouraud dextrose agar, grown at 25°C, were inoculated in brain heart infusion broth and incubated at 37°C. Fungal morphology of both yeast-like and mycelial phase was microscopically examined.

For molecular analysis, total genomic DNA was isolated by using the same extraction protocol as previously described in detail in Criseo and Romeo [13].

The protocol of Kano et al. [25], based on amplification of the partial chitin synthase 1 gene (Chs1-PCR), was used to confirm the identity of *S. schenckii*.

Amplification of the partial calmodulin-encoding gene was performed with primers CL1-GARTWCA AGGAGGCCTTCTC and CL2A-TTTTTGCATCAT GAGTTGGAC as described by O'Donnell et al. [26]. Following PCR, amplicons were purified with QIAquick PCR purification kit (Qiagen, Milan-Italy) and sequenced at MWG-biotech (M-Medical S.r.l, Milan, Italy) with the same primers used for PCR.

The identity of our nucleotide sequences was verified by BLASTN search (http:\\www.ncbi.nlm.nih.gov/blast). Several published *Sporothrix*-calmodulin-related sequences (Table 1) were retrieved from GenBank, and computer-assisted multiple sequence comparisons were performed using ClustalW algorithm implemented in MEGA4 software [27].

The multiple nucleotide sequence alignment was inspected, visually adjusted and subsequently was used for neighbor-joining analysis performed using MEGA4 software; confidence was estimated using 1,000 rounds of bootstrapping.

DNA and protein sequence analyses, to detect nucleotide and aminoacidic changes in the calmodulin exons, were performed with a combination of

Table 1 Strains, species, origin, and GenBank accession numbers of fungal isolates used in this study

Strains	Species[a]	Origin	Clade	GenBank
CDM18	*S. schenckii*	Clinical, Italy	IIa	This study
SPO1	*S. schenckii*	Clinical, Italy	IIa	This study
ScNS1 and ScNS2	*S. pallida*	Environmental, Italy	Va	This study
GER1, GER2, GER3, GER4	*S. pallida*	Environmental, Germany	Va	This study
SPA8	*S. pallida*	Environmental, Spain	Va	HQ686039[b]
SPA1, SPA2, SPA3	*S. pallida*	Environmental, Spain	Va	This study
ScCS1, ScCS2, ScCS3	*S. pallida*	Environmental, Italy	Vb	This study
ScMos	*S. pallida*	Environmental, Italy	Vb	This study
ScAs	*S. pallida*	Environmental, Italy	Vb	This study
SAM1, SAM2, SAM3, SAM4 and SAM5	*S. pallida*	Environmental, Austria	Vb	This study
BG	*S. pallida*	Environmental, Spain	Vb	This study
BG2	*S. pallida*	Environmental, Spain	Vb	This study
BG6	*S. pallida*	Environmental, Spain	Vb	HQ692915[b]
HOL1 and HOL2	*S. pallida*	Environmental, Holland	Vb	This study
HOL3	*S. pallida*	Environmental, Holland	Vb	HQ686040[b]
CBS 111110	*S. pallida*	Insect, Germany	Vb	AM398382
CBS 302.73T	*S. pallida*	Environmental, UK	Vb	AM398396
ATCC 18616	*S. luriei*	Clinical, South Africa	VI	AM747302
CBS 120342	*S. mexicana*	Environmental, Mexico	IV	AM398392
CBS 120341	*S. mexicana*	Environmental, Mexico	IV	AM398393
CBS 120340	*S. globosa*	Clinical, Spain	III	AM116908
IHEM 4178	*S. globosa*	Clinical, Italy	III	AM399018
FMR 9020	*S. globosa*	Clinical, Japan	III	AM398994
KMU 4116	*S. globosa*	Environmental, China	III	AM399019
KMU 4210	*S. globosa*	Environmental, China	III	AM399005
CBS 292.55	*S. globosa*	Clinical, UK	III	AM490354
KMU 4200	*S. globosa*	Environmental, China	III	AM399004
MCCL 220029	*S. globosa*	Clinical, India	III	AM490358
KMU 4208	*S. globosa*	Environmental, China	III	AM399002
UTHSC 04-1485	*S. globosa*	Clinical, USA	III	AM399015
FMR 8717	*S. schenckii*	Clinical, Peru	IIb	AM399017
IHEM 15499	*S. schenckii*	Clinical, Peru	IIb	AM117434
FMR 8716	*S. schenckii*	Clinical, Peru	IIb	AM399006
IHEM 15486	*S. schenckii*	Clinical, Peru	IIb	AM117432
FMR 8604	*S. schenckii*	Clinical, Peru	IIb	AM117429
IHEM 15477	*S. schenckii*	Clinical, Bolivia	IIa	AM117444
FMR 8679	*S. schenckii*	Clinical, Argentina	IIa	AM117445
IHEM 3774	*S. schenckii*	Clinical, Colombia	IIa	AM117447
KMU 975	*S. schenckii*	Clinical, Japan	IIa	This study
NBRC 8158	*S. schenckii*	Not Known, Japan	IIa	AM117438
CBS 359.36	*S. schenckii*	Clinical, USA	IIa	AM117437
CBS 938.72	*S. schenckii*	Clinical, France	IIa	AM490340
IPEC 17692	*S. brasiliensis*	Clinical, Brazil	I	AM159127
IPEC 17943	*S. brasiliensis*	Clinical, Brazil	I	AM116878
CBS 120339	*S. brasiliensis*	Clinical, Brazil	I	AM116899

Table 1 continued

Strains	Species[a]	Origin	Clade	GenBank
FMR 8337	*S. brasiliensis*	Environmental, Brazil	I	AM116876
IPEC 22582	*S. brasiliensis*	Clinical, Brazil	I	AM116891
IPEC 16864	*S. brasiliensis*	Clinical, Brazil	I	AM116889
IPEC 17585	*S. brasiliensis*	Clinical, Brazil	I	AM116887

[a] Identification based on calmodulin gene analysis

[b] The sequence was obtained in this study

Transeq, ClustalW and GeneWise computing packages (http:\\www.ebi.ac.uk).

Three representative nucleotide sequences: HQ686039 (strain SPA8; clade Va), HQ686040 (strain HOL3; clade Vb), and HQ692915 (strain BG6; clade Vb) have also been submitted to GenBank database.

Results

A total of 28 previously identified *S. schenckii* Italian strains (26 environmental and 2 clinical) [13] were examined in order to establish their genetic relatedness on the basis of the calmodulin sequences, the most phylogenetically informative locus found in recent studies [19, 20].

All *S. schenckii* isolates showed temperature dimorphism with the typical cigar-shaped form of the yeast-like cells grown at 37°C and distinctive micromorphology at 24°C. The identity of the fungus was further confirmed by Chs1-PCR that produced a DNA fragment of ~300 bp long from all tested strains including reference strains. These results point out that this molecular method does not discriminate among newly recognized *Sporothrix* species since it produces an identical DNA fragment independently from the species status of the examined strain (Fig. 1).

The amplified calmodulin genes yielded DNA fragments of approximatively 800 bp in size.

BLAST searches, using calmodulin sequences as query, revealed that all 26 environmental isolates had a high level of sequence similarity with *S. albicans* (99–100%), whereas the two clinical isolates showed high-score matches (99%) with *S. schenckii*.

The results of our phylogenetic analysis agree with those obtained in other previous studies where six well-defined and supported groups were found [19, 20] (Fig. 2).

The two Italian clinical strains were co-clustered with *S. schenckii* sensu stricto isolates; in particular, they fall within sub-clade IIa that includes *S. schenckii* isolates from different geographical origin, including both European strains: CBS 938.72 and CBS 359.36T [19] (Fig. 2).

Conversely, all 26 environmental isolates, significantly clustered (bootstrap confidence value of 100%) with clade V isolates (CBS 111110 and CBS 302.73) (Fig. 2), were previously shown to belong to *S. albicans* [19] whose name has been recently replaced by *S. pallida* [21]. Furthermore, our results

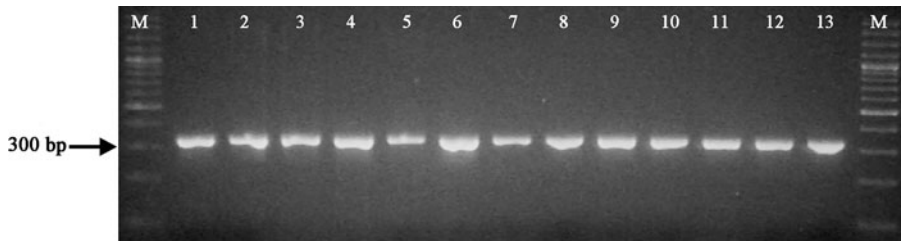

Fig. 1 Agarose gel electrophoresis of DNA fragments obtained by amplification of the CHS1 gene. *M* molecular size marker; *Lanes 1–3 S. schenckii* ATCC 10268, CDM18 and SPO1, respectively; *Lane 4 S. luriei* KMU 2787; *Lane 5 S. mexicana* FMR 9108; *Lane 6 S. brasiliensis* SS74; *Lane 7 S. globosa* SS49; *Lane 8 S. pallida* CBS 302.73; *Lanes 9–13* Italian environmental isolates: GER1, SPA1, SAM1, BG, and HOL3, respectively

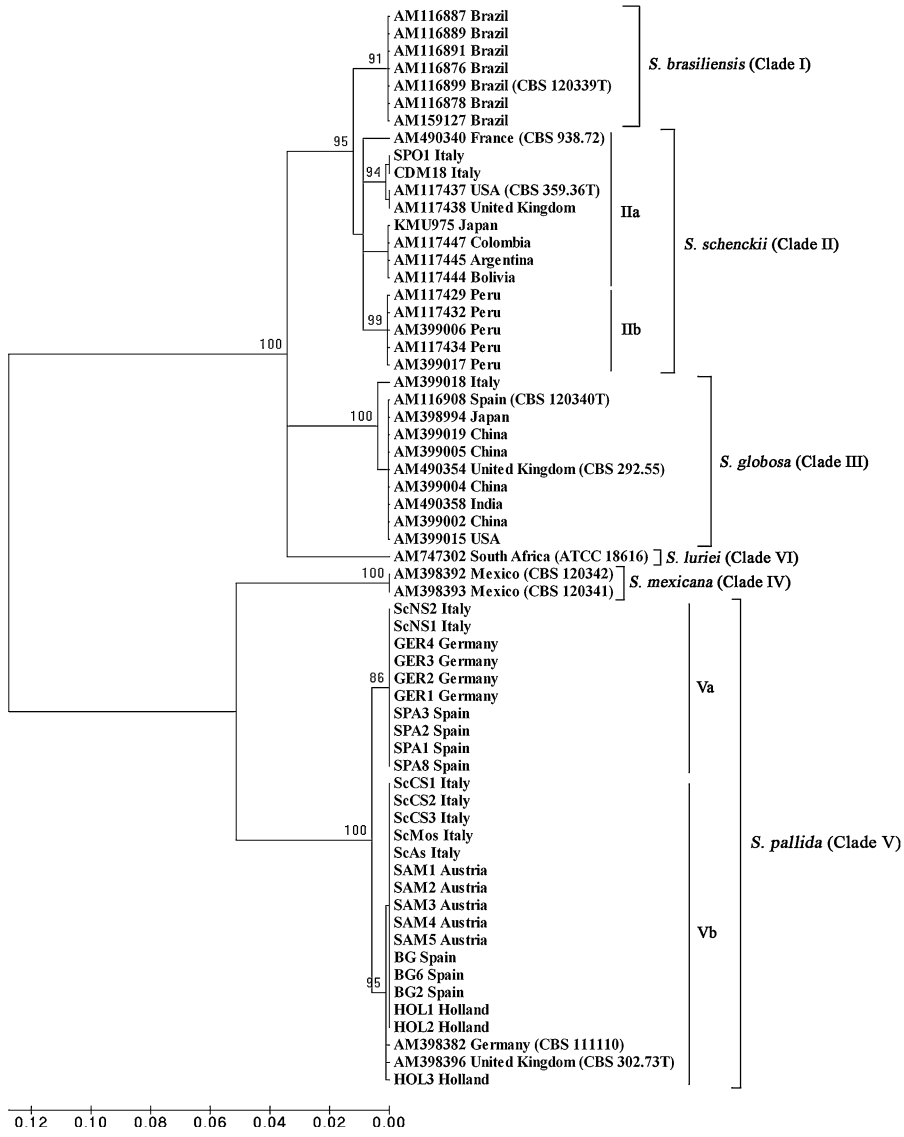

Fig. 2 Phylogenetic tree generated by neighbor-joining analysis using partial nucleotide sequences of the calmodulin-encoding gene. Bootstrap support values above 85% are indicated at the nodes

showed that clade V could be further subdivided into two sub-clades, designated Va and Vb, consisting of fungal isolates from different European countries (Fig. 2). More precisely, 8 of 10 *S. pallida* strains of sub-clade Va were previously isolated from commercial amended soil purchased in Italy, but produced and packaged in Spain (4 isolates), Germany (4 isolates) and imported; the remaining two isolates were found in natural soil in Reggio Calabria, Italy [13]. The 16 isolates of sub-clade Vb were, indeed, recovered from environmental and commercial samples from Italy (5 isolates) and commercial soils imported from Austria (5 isolates), Spain (3 isolates), and Holland (3 isolates) [13]. This latter group (Vb) was found to be more heterogeneous than the sub-clade Va, suggesting that these two sub-clades, which contain three different types of strains, could likely correspond to the previously synonymized species: *S. pallida*, *S. nivea,* and *S. albicans*. Unfortunately, it was not possible for us to test this hypothesis because of the lack of published calmodulin-related sequences from these species. In fact specific searches in GenBank database revealed only the presence of ribosomal and β-tubulin sequences, which have been previously used as genetic markers to evidence the synonymy of these fungi [21].

Bioinformatic analysis revealed that genetic differences between members of the two sub-clades were due to mutations located mostly in intronic regions; only isolates of sub-clade Vb, except the two reference isolates and HOL3 strain (Fig. 2) showed a silent mutation (Arg74; CGC → CGT) in the third exon of the calmodulin.

Discussion

In past years, several molecular studies have clearly demonstrated that *S. schenckii* isolates displayed genetic characteristics so different that they appear not to be the same species [16, 28–31]. Hereafter, the taxonomy of *S. schenckii* has undergone significant changes due to the description of new closely related cryptic species [19, 20]. In these cases, as it has happened for other clinically relevant pathogenic fungi [32–34], retrospective and prospective epidemiological studies are necessary to better know the incidence and clinical significance of these new species. However, it remains difficult, nowadays, to identify correctly the members of the *S. schenckii* complex in clinical laboratories due to lack of rapid and specie-specific molecular methods. In fact, the results obtained here, and in our previous study [13], indicate that the amplification of the chitin synthase gene [25] as well as that of the DNA topoisomerase II gene [35] produces identical DNA patterns in *S. schenckii* sensu stricto and its related species; hence, these methods are not reliable tests for discrimination of members of the *S. schenckii* group. In effect, our data suggest that all molecular methods, for specific identification of *S. schenckii,* developed before the recent description of the new *Sporothrix* species should be used with caution because the proper identification of the species causing the infection is needed in order to choose the most effective antifungal treatment. These observations are strongly supported by the recent study of Marimon et al. [24] who examined 92 isolates belonging to the *S. schenckii* complex and found that there were significant differences in antifungal susceptibilities between species tested. Therefore, only sequencing of highly informative genetic loci such as the calmodulin-encoding gene is, at present, useful for elucidating relationships and differentiates among species of the *S. schenckii* complex.

Although in general, ribosomal DNA genes evolve cohesively within a single species and exhibit normal levels of sequence divergence between rDNA copies from different species, in the case of *S. schenckii* group, the use of this genetic marker could lead to wrong conclusions as already happened in other studies [13, 16, 21].

The inefficacy of ITS regions for resolving the phylogeny of *S. schenckii* has already been demonstrated by de Meyer et al. [21]. In their study, these authors reclassified some previous environmental *S. schenckii* strains [16] by using sequencing of the β-tubulin gene because genetic data based on ITS sequences alone were unable to resolve phylogenetic relationship among the strains [16, 21]. Likewise, in our previous phylogenetic study based on sequencing of the D1-D2 region of 28S rDNA, we have found two strongly supported groups: one group composed entirely of isolates of clinical origin and a second one containing only environmental isolates [13]. The observed genetic differences, between these two groups, led us to hypothesize the presence of cryptic related species that has been demonstrated in this study. In fact, all 26 members of the environmental group [13] have, here, been identified as *S. pallida*. The exclusive presence of this non-pathogenic species in our environmental samples, as well as the absence of *S. schenckii* sensu stricto strains, may explain in part why the sporotrichosis has a low incidence in Italy and in Europe in general.

Interestingly, in this study, the *S. pallida* (clade V) was found to be quite heterogeneous and was further subdivided into two subgroups (Va and Vb). Bioinformatic analysis revealed that the genetic differences between members of these groups were due to nucleotide substitutions mainly located within intronic regions (near exon/intron junctions). However, all isolates of the subgroup Vb, except CBS 111110, CBS 302.73T and HOL3 strains, also showed one characteristic nucleotide transition (C → T) in the third exon of the calmodulin, but this mutation was silent and not accompanied by any amino acid alteration.

Finally, only two isolates were recognized as *S. schenckii* sensu stricto, and this was consistent with their origin and their pathogenic nature; in fact, these strains represent the only examples of authentic clinical *S. schenckii* isolates from Italy.

In conclusion, the data reported in this study not only suggest further environmental and clinical

investigations to reveal the occurrence of these newly described *Sporothrix* species in Europe but also bring to our attention the need to develop rapid and specific molecular methods to differentiate these new *Sporothrix* species in the clinical laboratories.

Acknowledgments We thank Prof. Zoilo Pires De Camargo (Department of Microbiology, Immunology and Parasitology, Federal University of São Paulo, Brazil) for supplying *S. brasiliensis* SS74 and *S. globosa* SS49 strains; we are also grateful to Prof. Masako Kawasaki (Department of Dermatology, Kanazawa Medical University, Ishikawa, Japan) for generously providing us the strains: *S. schenckii* KMU975 and *S. luriei* KMU 2787 and for useful discussions. We also thank Prof. Josep Guarro (Unitat de Microbiologia, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Reus, Spain) and Prof. Claudia Cafarchia (Faculty of Veterinary Medicine, University of Bari, Italy) for sending us the strains of *S. mexicana* FMR 9108 and *S. schenckii* CDM18, respectively.

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