Genetic diversity and antifungal susceptibility profiles in agents of sporotrichosis

Messias Rodrigues, A.; de Hoog, G.S.; de Cássia Pires, D.; Nogueira Brihante, R.S.; da Costa Sidrim, J.J.; Gadelha, M.F.; Lopes Colombo, A.; Pires de Camargo, Z.

Published in:
BMC Infectious Diseases

DOI:
10.1186/1471-2334-14-219

Citation for published version (APA):
Messias Rodrigues, A., de Hoog, G. S., de Cássia Pires, D., Nogueira Brihante, R. S., da Costa Sidrim, J. J., Gadelha, M. F., Lopes Colombo, A., & Pires de Camargo, Z. (2014). Genetic diversity and antifungal susceptibility profiles in agents of sporotrichosis. BMC Infectious Diseases, 14, 219. https://doi.org/10.1186/1471-2334-14-219
Genetic diversity and antifungal susceptibility profiles in causative agents of sporotrichosis

Anderson Messias Rodrigues1, G Sybren de Hoog2, Débora de Cássia Pires3, Raimunda Sámia Nogueira Brihante4, José Júlio da Costa Sidrim4, Marcos Fabio Gadelha5, Arnaldo Lopes Colombo3 and Zoilo Pires de Camargo1*

Abstract

Background: Sporotrichosis is a chronic subcutaneous mycosis of humans and animals, which is typically acquired by traumatic inoculation of plant material contaminated with Sporothrix propagules, or via animals, mainly felines. Sporothrix infections notably occur in outbreaks, with large epidemics currently taking place in southeastern Brazil and northeastern China. Pathogenic species include Sporothrix brasiliensis, Sporothrix schenckii s. str., Sporothrix globosa, and Sporothrix luriei, which exhibit differing geographical distribution, virulence, and resistance to antifungals. The phylogenetically remote species Sporothrix mexicana also shows a mild pathogenic potential.

Methods: We assessed a genetically diverse panel of 68 strains. Susceptibility profiles of medically important Sporothrix species were evaluated by measuring the MICs and MFCs for amphotericin B (AMB), fluconazole (FLC), itraconazole (ITC), voriconazole (VRC), posaconazole (PCZ), flucytosine (5FC), and caspofungin (CAS). Haplotype networks were constructed to reveal interspecific divergences within clinical Sporothrix species to evaluate genetically deviant isolates.

Results: ITC and PCZ were moderately effective against S. brasiliensis (MIC90 = 2 and 2 μg/mL, respectively) and S. schenckii (MIC90 = 4 and 2 μg/mL, respectively). PCZ also showed low MICs against the rare species S. mexicana. 5FC, CAS, and FLC showed no antifungal activity against any Sporothrix species. The minimum fungicidal concentration ranged from 2 to >16 μg/mL for AMB against S. brasiliensis and S. schenckii, while the MFC90 was >16 μg/mL for ITC, VRC, and PCZ.

Conclusion: Sporothrix species in general showed high degrees of resistance against antifungals. Evaluating a genetically diverse panel of strains revealed evidence of multidrug resistant phenotypes, underlining the need for molecular identification of etiologic agents to predict therapeutic outcome.

Keywords: Sporothrix schenckii, Sporothrix brasiliensis, Multidrug resistance, MIC, MFC, Intraspecific diversity

Background

Sporotrichosis is a (sub)cutaneous mycosis of man and animals that is highly prevalent in tropical and subtropical areas, and currently reaching epidemic proportions in Brazil [1-3] and China [4,5]. Over the last decade, sporotrichosis has changed from a relatively obscure endemic infection to an epidemic zoonotic health problem in the South and Southeast Regions of Brazil [2]. Infection typically occurs by cutaneous inoculation of the microorganism from environmental sources [6,7] or via zoonotic transmission [3]. Sporotrichosis is a chronic disease that can spread lymphatically and most often presents as a localized lesion with subcutaneous nodules that eventually break through the skin [7,8]. Although rare, hematogenous dissemination can also occur depending on the immune status of the host [9].

Recent studies on molecular phylogeny clarified species boundaries within clinical isolates of Sporothrix and provided strong support for many cryptic groups. The major causative agents of sporotrichosis are several closely related thermomorphic fungi of the genus Sporothrix [10,11], including Sporothrix brasiliensis, Sporothrix schenckii s. str., Sporothrix globosa, and Sporothrix luriei [2,5]. These species have distinct virulence profiles [12],...
karyotypes [13] and geographic distributions ranging from regional [1,10] to global [5]. The *Sporothrix* genus, embedded in the plant-associated order Ophiostomatales, exhibits high genetic diversity [10,11], which is accompanied by a diversity of *in vitro* responses to the main antifungal agents [14] used in sporotrichosis treatment [7,15]. Such agents include potassium iodide [16] and itraconazole [17] for localized cutaneous and lymphocutaneous forms, and amphotericin B for disseminated cases [6,9,16]. Screening for antifungal susceptibilities during epidemiological surveillance programs could help to uncover putative multidrug resistant *Sporothrix* strains, and improve our ability to adjust therapeutic regimens and reduce relapse.

Despite the considerable spread of the Brazilian epidemic of sporotrichosis over the last two decades [1-3,10] and the increased occurrence of atypical cases [9], no study of this illness to date has integrated molecular epidemiology and antifungal drug susceptibility. The present study aimed to evaluate the resistance profiles to currently used antifungals in a genetically diverse panel of *Sporothrix* strains representing the main pathogenic species.

**Methods**

**Fungal isolates**

This study included 68 *Sporothrix* isolates that were obtained from clinical lesions of patients with varying degrees of disease severity (n = 65 human/n = 2 animal) or from environmental sources (n = 1). The isolates were originally received as *S. schenckii s.l.*, and were subsequently identified down to the molecular species level by Rodrigues *et al.* [12,19] (Additional file 1: Table S1). Isolates were stored at room temperature in slant cultures on Sabouraud dextrose agar (SDA; Difco Laboratories, Detroit, MI). Ethical approval was provided by Institutional Committee (UNIFESP-0244/11).

**Antifungal agents and susceptibility testing**

Culture conditions and inoculum preparation were as described by Marimon *et al.* [14]. Using the methods recommended in the CLSI standard document M38-A2, we evaluated minimum inhibitory concentrations (MIC) for the following seven antifungal agents: amphotericin B (AMB), fluconazole (FLC), itraconazole (ITC), voriconazole (VRC), posaconazole (PCZ), flucytosine (5FC), and caspofungin (CAS). The final drug concentrations ranged from 0.03 to 16 μg/mL for AMB, ITC, VRC, PCZ, and CAS, and from 0.12 to 64 μg/mL for 5FC and FLC. The microplates were incubated at 35°C and read after 72 h. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality controls for the experiments.

To obtain the minimum fungicidal concentration (MFC), for each serial dilution, 10 μL was taken from each well with no visible growth and spread on Sabouraud dextrose agar (Difco Laboratories, USA). Plates were then incubated at 28°C for 48–72 h. The MFC was defined as the lowest drug concentration that yielded three or fewer colonies, i.e., 95.5 to 99% killing activity [20]. The Mann–Whitney U test was used to determine the distribution of MIC and MFC data. A p value of <0.05 was considered to indicate statistical significance.

**Molecular characterization**

The isolates were genetically characterized [1,10] based on calmodulin gene sequencing using the primers CL1 (5′-GAR TWC AAG GAG GCC TTC TC) and CL2A (5′-TTT TTG CAT CAT GAG TTG GAC) [21], as well as sequencing part of the rRNA operon [5] using the primers ITS1 (5′-TCC GTA GGG GAA CCT TGC GG) and ITS4 (5′-TCC TTC GCT TAT TGA TAT GC) [22]. We choose these loci because they have previously been extensively used for phylogeny and taxonomy of this complex of cryptic species [1-3,5,10,11,23]. To increase the quality of sequence data, sense and antisense sequences were determined with an ABI 3730 DNA Analyzer (Applied Biosystems, Inc., Foster City, CA, USA) and thereafter assembled into single sequences via CAP3 using bases with a *Phred* quality score of ≥30. All sequences were deposited online at GenBank (Additional file 1: Table S1).

Phylogenetic histories were reconstructed using maximum likelihood (ML) and neighbor-joining (NJ) methods with the Mega5 software [24], using 1,000 bootstrap replicates to estimate the confidence values for individual branches [25]. Evolutionary distances were calculated using the Tamura 3-parameter model [26]. The rate of change between sites was modeled with a gamma distribution (shape parameter = 1). Nucleotide (n) and haplotype (Hd) diversities [27] were estimated using DnaSP software version 5.10 [28]. Haplotype networks were used to visualize differences and diversity among *S. brasiliensis* and *S. schenckii* sequence data [28]. Gaps and missing data were excluded in the calculations. Median-joining networks [29] for the concatenate dataset (*CAL* + *ITS*) were obtained and visualized using the Network 4.610 software, as previously described by Rodrigues *et al.* [3].

**Results**

The combined dataset (*CAL* + *ITS*) yielded a sequence alignment of 1,397 positions, including 866 invariable characters, 232 variable parsimony-informative sites (16.6%), and 185 variable singletons. The topologies of the ML and NJ trees were congruent (I_congr = 3.66; p = 1.68 e-24) [30] and the isolates were distributed among the
six major clades (Figure 1), in agreement with previous studies [1-3,10,11].

Table 1 summarizes the MIC and MFC ranges for the human clinical Sporothrix isolates. Sporothrix brasiliensis exhibited low genetic diversity (π = 0.00194), and comprised seven haplotypes (H1–H7). Of the tested drugs, the triazoles ITC and PCZ showed the best activity against S. brasiliensis strains, with MICs ranging from 0.25 to 4 μg/mL (Figure 2A) and from 0.5 to 2 μg/mL (Figure 2C), respectively. The MICs for ITC were lower in S. brasiliensis compared to in S. schenckii (p = 0.0001) and S. mexicana (p = 0.0031). We detected moderate activity for the polyene AMB against S. brasiliensis strains, with MICs ranging from 1 to 8 μg/mL (Figure 2E), while CAS, 5FC, FLC, and VRC showed poor activity.

Sporothrix schenckii represented by the genetically heterogeneous clade II (π = 0.00917) was divided into ten haplotypes (H8–H18) and showed a broad MIC range for the different antifungals. ITC (Figure 2A) and PCZ (Figure 2C) presented good activity against most S. schenckii s. str. strains, with MICs ranging from 0.25 to >16 μg/mL and from 0.06 to >16 μg/mL, respectively. The MICs for PCZ were slightly higher in S. schenckii compared to in S. brasiliensis (p = 0.0076). AMB presented

![Figure 1 Phylogenetic relationships of calmodulin-encoding gene and ITS sequences among clinical and environmental Sporothrix species.](http://www.biomedcentral.com/1471-2334/14/219)
| Species                  | AMB (µg/mL) |        | FLC (µg/mL) |        | ITC (µg/mL) |        | VRC (µg/mL) |        | PCZ (µg/mL) |        | SFC (µg/mL) |        | CAS (µg/mL) |        |
|-------------------------|-------------|--------|-------------|--------|-------------|--------|-------------|--------|-------------|--------|-------------|--------|-------------|--------|
|                         | MIC        | MFC    | MIC        | MFC    | MIC        | MFC    | MIC        | MFC    | MIC        | MFC    | MIC        | MFC    | MIC        | MFC    |
| *S. brasiliensis* (n = 22) | Range      | 1 - >16| >64       | >64    | 0.25 - 4   | 1 - >16| 2 - >16     | 16 - >16| 0.5 - 2    | 1 - >16| 64 - >64   | >64    | >16        | >16    |
| 50%                     | 4          | 4      | >64       | >64    | 1          | 16     | 16          | >16    | 1          | >16    | 64          | >64    | >16        | >16    |
| 90%                     | 4          | 16     | >64       | >64    | 2          | >16    | >16         | >16    | 2          | >16    | >64         | >64    | >16        | >16    |
| *S. schenckii* (n = 39) | Range      | 1 - >16| >64       | >64    | 0.25 - >16 | 0.5 - >16| 4 - >16    | 4 - >16| 0.06 - >16 | 1 - >16| 64 - >64   | >64    | >16        | >16    |
| 50%                     | 8          | 16     | >64       | >64    | 1          | 16     | 16          | >16    | 1          | >16    | 64          | >64    | >16        | >16    |
| 90%                     | >16        | >16    | >64       | >64    | 4          | >16    | >16         | >16    | 2          | >16    | >64         | >64    | >16        | >16    |
| *S. globosa* (n = 4)    | Range      | 2 - >16| >64       | >64    | 0.5 - >16  | 0.5 - >16| 2 - 8      | 8 - >16| 0.5 - 16   | 1 - >16| 8 - >64     | >64    | 8 - >16    | >16    |
| 50%                     | -          | -      | -         | -      | -          | -      | -          | -      | -          | -      | -          | -      | -          | -      |
| 90%                     | -          | -      | -         | -      | -          | -      | -          | -      | -          | -      | -          | -      | -          | -      |
| *S. mexicana* (n = 3)   | Range      | 8 - >16| >64       | >64    | 2 - >16    | 2 - >16| 1 - >16    | 16 - >16| 1 - >16    | 16 - >16| 64 - >64   | >64    | 8 - >16    | >16    |
| 50%                     | -          | -      | -         | -      | -          | -      | -          | -      | -          | -      | -          | -      | -          | -      |
| 90%                     | -          | -      | -         | -      | -          | -      | -          | -      | -          | -      | -          | -      | -          | -      |
| Overall (n = 68)        | Range      | 1 - >16| >64       | >64    | 0.25 - >16 | 0.5 - >16| 1 - >16    | 4 - >16| 0.06 - >16 | 1 - >16| 8 - >64     | >64    | 64 - >64   | >16    |
| 50%                     | 4          | 16     | >64       | >64    | 1          | >16    | 16          | >16    | 1          | >16    | 64          | >64    | >16        | >16    |
| 90%                     | >16        | >16    | >64       | >64    | >16        | >16    | >16         | >16    | 2          | >16    | >64         | >64    | >16        | >16    |
Figure 2 (See legend on next page.)

H1-H7: *S. brasiliensis* (Clade I)
H8-H14: *S. schenckii* (Clade II)
H19: *S. junin* (Clade VI)
H20: *S. globosa* (Clade III)
H21: *S. mexicana* (Clade VI)
H22: *S. paillida* (Clade V)
H23: *S. bruneoviolacea*
Figure 2 Genetic diversity and antifungal susceptibility profiles in causative agents of sporotrichosis. Median-joining haplotype network of Sporothrix schenckii complex isolates based on calmodulin and ITS concatenated sequences. The circumference size is proportional to the haplotype frequency. Black dots (median vectors) represent unsampled or extinct haplotypes in the population. Mutational steps are represented by white dots and, in cases of long branches, by values. The charts represent the MIC (A, C, and E) and MFC (B, D, and F) distributions within the population for ITC, PCZ, and AMB.

Discussion
Given the high intrinsic antifungal resistance in Sporothrix, studies correlating antifungal susceptibility and genetic diversity among etiological agents of sporotrichosis are overdue. Here we used haplotype networks to estimate genetic diversity among clinical Sporothrix species. This approach has the strength of enabling the recognition of genetically deviating strains in a population, rather than randomly sampled frequent haplotypes with similar genetic background, as represented by the frequent haplotypes H1 and H8 in the S. brasiliensis and S. schenckii populations, respectively. However, haplotype-based strategies are dependent on the molecular marker used to estimate diversity, the set of strains comprising the database as well as the network methods [31]. To date, the partial sequence of CAL and ITS region (ITS1/2 + 5.8 s) has been used to estimate genetic diversity and taxonomy of clinical Sporothrix species [2,5,10,11]. Indeed, due to its highly discriminatory power and polymorphisms we choose these regions to give insights into the genetic diversity in sympatric populations of S. brasiliensis and S. schenckii s. str.

A genetically homogeneous population may be expected to have similar susceptibility profiles among the individuals, but increased fitness during population expansion can result in a diversity of susceptibility profiles. Alternatively, differential responses may emerge over time in ancestral genetically diverse species. Of the phylogenetically related species S. brasiliensis and S. schenckii s. str., the former has a lower genetic diversity [2,3,10,13], and here we found that it also had a correspondingly lower variability of in vitro susceptibility. A larger degree of genetic diversity has been observed among S. schenckii s. str. isolates, which is accompanied by greater variation in antifungal profiles [14] virulence [12] and genomic organization [13]. Moreover, the presence of median vectors (mv1-3, 5, 6) in the haplotype network (Figure 2) may indicate extinct or intermediate unsampled haplotypes within the S. schenckii s. str. populations, possibly suggesting the existence of even more variation. Conversely, the absence of median vectors in the S. brasiliensis population strongly suggests that our analysis detected most of the strain diversity circulating in the human epidemic, thus supporting the low variation of the MIC and MFC values in this species.

Itraconazole is the drug of choice for treating endemic mycoses caused by thermomorph fungi, including sporotrichosis. Our findings regarding the in vitro activities of the main antifungal drugs are in agreement with previous studies, indicating that ITC and PCZ were moderately effective against isolates of S. brasiliensis and S. schenckii s. str. [14,32]. Despite the relatively high values found in all studies, several authors have demonstrated the efficacy of ITC in clinical outcome. Barros et al. [17] studied the clinical treatment of a large number of patients in Rio de Janeiro, and reported the success of ITC treatment (50–400 μg/day) in 94.6% patients (n = 610). Although this study did not molecularly identify the phylogenetic species involved, it was most likely S. brasiliensis, as this species is highly prevalent in Rio de Janeiro due to feline sporotrichosis outbreaks [1-3,10]. Such an epidemiological profile could explain the favorable results for ITC. The relatively low MICs found for PCZ also suggest this molecule to be a promising drug in the treatment of sporotrichosis caused by S. schenckii s. str. or S. brasiliensis. Additionally, PCZ also showed low MICs against 2 out of 3 clinical isolates of the rare species S. mexicana, including CBS 132928 (MIC = 1 μg/mL) and CBS 132927 (MIC = 2 μg/mL). Only a few strains of S. mexicana have been described in the literature [1,10], and the species has been reported as tolerant to most commercially available drugs [14,33].

Resistance to amphotericin B has been reported in emerging pathogens, such as Aspergillus terreus, Candida lusitaniae, Fusarium spp., Scedosporium prolificans,
**Trichosporon asahii** [34,35]. Here we found high MICs for AMB in five isolates of *S. schenckii s. str.* (Ss17, Ss22, Ss110, and Ss119 from H8, and isolate Ss51 from H12) with MIC and MFC values of >16 μg/mL. The multidrug resistant phenotype found for isolate Ss51 (H12; Figure 2B, D, and F) is particularly noteworthy in relation to its clinical origin, where it showed moderate virulence in a murine model [12]. The high MICs found for the remaining compounds (FLC, VRC, CAS, and 5FC) are in agreement with previous reports [14,32,36].

Fungicidal drugs are capable of inhibiting the cell growth and reproduction of fungi without destroying them. Drug-resistant pathogens may evolve resistance under the selective pressure imposed by continuous exposure to fungistatic drugs [37]. The molecular mechanism that lies behind the recent emergence of drug-resistant phenotype among *Sporothrix* species is currently unknown. However, judging from other fungi, the increased and prolonged use of triazoles has raised concerns about resistant infections by *Cryptococcus neoformans* [38], *Candida albicans* [39] and *Aspergillus fumigatus* [40]. Azole resistance may occur through a diversity of mechanisms including the upregulation of multidrug transporter genes that leads to enhanced efflux of azoles and therefore reduce drug accumulation [41]; multiple genetic alterations of the target enzyme that can affect the affinity of the enzyme and therefore prevents azole binding [42], and alteration of metabolism, usually sterol synthesis [43]. More recently, genetic studies correlating antifungal resistance and alteration in chromosome copy number and genomics architecture has delivered an exciting view on the molecular mechanisms governing the increased fitness to the phenotype of azole-resistance [38,44-46].

*Candida albicans* [39,44,45] and *Cryptococcus neoformans* [38,46,47] triazole-resistant isolates very often contain an abnormal number of chromosomes. Such genomic plasticity may offer additional copies of drug resistance genes leading chromosomal aneuploidies isolates to overcoming the drug effects [44,45] and allowing rapid adaptive evolution [47]. Although this phenomenon may occur spontaneously [47], drug-resistant aneuploidies arise very frequently within drug-exposed fungal populations and this may support our recent findings on chromosomal polymorphisms in *Sporothrix* species [13]. The intra-specific polymorphisms in chromosome number and size in *S. schenckii s. str.* suggested that aneuploidy occur among clinical isolates, and it is tempting to hypothesize that this phenomenon could, in part, be responsible for the differences in drug profiles observed here. Therefore, the high karyotype diversity observed for *S. schenckii s. str.* [13] is reflected in the genetic diversity found in our haplotype network (Figure 2). However, testing for this pattern requires a larger number of isolates with dissimilar antifungal profiles as well as chromosomal polymorphism.

Fungicidal drugs may be defined as those that lead to a reduction of 99.9% of the initial inocula [48]. It is difficult to make comparisons between published MFCs studies, since most were performed before the introduction of clinical species beyond *S. schenckii s. str.* to the genus *Sporothrix*. Two or more of these newer species were likely involved in the studies of McGinnis et al. [49] and Silveira et al. [50]. In all clinical *Sporothrix* spp. evaluated, we found significant differences between the minimum concentrations needed to inhibit fungal cell growth and the concentration required for colony count reduction. In all studied species, most isolates were able to grow after 72 h in the presence of each drug at the maximum concentration tested, which is in agreement with results obtained by Trilles et al. [36]. The moderately low MICs for triazoles imply a great resistance to killing among isolates of *S. brasiliensis*, the most pathogenic among *Sporothrix* species.

Spontaneous cure, as well as relapse, are common features of sporotrichosis. Our present data show that most antifungal agents had only a fungistatic effect against clinical isolates. It remains unclear whether there is a correlation between *in vitro* MIC/MFC data and clinical outcome in human sporotrichosis. Experimental murine models have shown the efficacy of posaconazole (5 mg/kg) for treating *S. brasiliensis* and *S. schenckii s. str.* infections [51]. Voriconazole (40 mg/kg) has been demonstrated to only reduce fungal load in mice infected with *S. schenckii s. str.*, and to have no activity against *S. brasiliensis* [52]. No studies to date have linked the dissimilar phylogenetic species to *in vitro* and *in vivo* data. Our *in vitro* results for the highly tolerant strain CBS 133020 (=Ss265, haplotype 7), an isolate originated from a disseminated case of *S. brasiliensis* in an HIV patient [9], shown that despite ITC and AMB MICs of 1 and 4 μg/mL, respectively, and MFCs of >16 and 8 μg/mL, respectively, the patient showed positive clinical outcome after administration of intravenous AMB (including 10 days on L-AMB) [9], indicating a need to monitor MFCs values before and during treatment.

**Conclusions**

Our study presents antifungal susceptibility profiles using MIC and MFC data, which is particularly noteworthy in light of recent taxonomic changes in medically relevant *Sporothrix* species. We assessed a genetically diverse set of strains with varying degrees of disease severity (fixed cutaneous, lymphocutaneous, or disseminated), and the antifungal susceptibility assays revealed a broad spectrum for the triazoles (ITC, PCZ, and VRC). This finding may have implications for the choice of antifungal therapy in different epidemiological risk groups, potentially having a substantial impact on clinical outcome. Furthermore, our data indicate the existence of multidrug resistant strains within
the \textit{S. schenckii} complex, underlining the need for con-
tinuous susceptibility screening. Further studies should be
directed to connect in vitro and clinical data, as well as
to investigate the molecular mechanisms underlying the
different resistance profiles among \textit{S. schenckii} and its
relatives.

\section*{Additional file 1: Table S1.}
Strains, species, origin, haplotypes, and
GenBank accession numbers (CAL and ITS) for the \textit{Sporothrix} spp. isolates
used in this study and the reference strains.

\section*{Abbreviations}
ITS: Internal transcribed spacer; CAL: Calmodulin; MICs: Minimum inhibitory
concentration; MFC: Minimum fungidial concentration; AMB: Amphotericin
B; FLC: Fluconazole; ITC: Itraconazole; VRC: Voriconazole; PCZ: Posaconazole;
\textit{S. globosa} sensu stricto; \textit{S. brasiliensis} sensu stricto; \textit{S. globosa} sensu lato;
ML: Maximum likelihood; NJ: Neighbor-joining; mV: Median vector.

\section*{Competing interests}
The authors declare that they have no competing interests. The authors alone
are responsible for the content and writing of the paper.

\section*{Authors' contributions}
AMR and DCP performed the experiments. AMR, ALC, and ZPC designed the
experiments. AMR, GSDH, RSNB, JICS, MFG, ALC, and ZPC analyzed the data,
drafted the manuscript together. All authors read and approved the final
manuscript.

\section*{Acknowledgements}
The authors are thankful for funding from Fundação de Amparo à Pesquisa
do Estado de São Paulo (FAPESP 2009/54024-2) and National Council for
Scientific and Technological Development (CNPq 472600/2011-7). AMR
(FAPESP 2011/07350-1) is a fellow of FAPESP.

\section*{Author details}
1Department of Microbiology, Immunology, and Parasitology, Cellular Biology
Division, Federal University of São Paulo (UNIFESP), São Paulo, SP, Brazil.
2Centralimpe para Schimmencultures, KNIAW Fungal Biodiversity Centre,
Utrecht, The Netherlands. 3Department of Medicine, Infectious Diseases
Section, Federal University of São Paulo (UNIFESP), São Paulo, SP, Brazil.
4Specialized Medical Mycology Center, Postgraduate Program in Medical
Microbiology, Federal University of Ceará, Fortaleza, Ceará, Brazil.
5Postgraduate Program in Veterinary Science, State University of Ceará,
Fortaleza, Ceará, Brazil.

Received: 11 January 2014 Accepted: 10 April 2014
Published: 23 April 2014

\section*{References}
1. Rodrigues AM, de Hoog S, de Camargo ZP: Emergence of pathogenicity in
the \textit{Sporothrix schenckii} complex. Med Mycol 2013, 51:405–412.
2. Rodrigues AM, de Hoog GS, Zhang Y, Camargo ZP: Emerging
sporotrichosis is driven by clonal and recombinant \textit{Sporothrix} species.
Emerg Microbes Infect 2014, 3:e32.
3. Rodrigues AM, de Melo TM, de Hoog GS, Schubach TMP, Pereira SA,
Fernandes GF, Bezerra LML, Felipe MS, de Camargo ZP: Phylogenetic
analysis reveals a high prevalence of \textit{Sporothrix brasiliensis} in feline
sporotrichosis outbreaks. PLoS Negl Trop Dis 2013, 7:e2281.
4. Song Y, Li SS, Zhong SX, Liu YY, Yao L, Huo SS: Report of 457
sporotrichosis cases from Jilin province, northeast China, a serious
endemic region. J Eur Acad Dermatol Venereol 2013, 27:313–318.
5. Zhou X, Rodrigues AM, Feng P, Hoog GS: Global ITS diversity in the
\textit{Sporothrix schenckii} complex. Functal Divers 2013, 1–13.
doi:10.1007/s13225-013-0220-2.
6. Kauffman CA, Bustamante B, Chapman SW, Pappas PG: Clinical practice
guidelines for the management of sporotrichosis: 2007 update by the
Infectious Diseases Society of America. Clin Infec Dis 2007, 45:1255–1265.
7. Kauffman CA: Sporotrichosis. Clin Infect Dis 1999, 29:231–237.
8. Bonfaz A, Vázquez-González D: Diagnosis and treatment of
lymphocutaneous sporotrichosis: What are the options? Curr Fungal
Pathol Rep 2013, 2:252–259.
9. Silva-Vergara ML, de Camargo ZP, Silva PF, Abdalla MR, Sgarbieri RN,
Rodrigues AM, dos Santos KC, Barata CH, Ferreira-Paim K: Disseminated
\textit{Sporothrix brasiliensis} infection with endocardial and ocular involvement
in an HIV-infected patient. Am J Trop Med Hyg 2012, 86:377–480.
10. Marimon R, Cano J, Gené J, Sutton DA, Kawakai M, Guarro J: \textit{Sporothrix}
brazilensis, \textit{S. globosa}, and \textit{S. mexicana}, three new \textit{Sporothrix} species of
clinical interest. J Clin Microbiol 2007, 45:3198–3206.
11. Marimon R, Gené J, Cano J, Trilles L, Dos Santos Lazera M, Guarro J:
Molecular phylogeny of \textit{Sporothrix schenckii}. J Clin Microbiol 2006,
44:3251–3256.
12. Fernandes GF, dos Santos PO, Rodrigues AM, Sasaki AA, Burger E, de
Camargo ZP: Characterization of virulence profile, protein secretion and
immunogenicity of different \textit{Sporothrix schenckii} sensu stricto isolates
compared with \textit{S. globosa} and \textit{S. brasiliensis} species. Virulence 2013,
4:241–249.
13. Sasaki AA, Fernandes GF, Rodrigues AM, Lima FM, Marini MM, dos Feitosa
SL, de Melo Teixeira M, Felipe MSS, da Silveira JF, de Camargo ZP:
Chromosomal polymorphism in the \textit{Sporothrix schenckii} complex. PLoS ONE
2014, 9:e88619.
14. Marimon R, Serena C, Gené J, Cano J, Guarro J: In vitro antifungal
susceptibilities of five species of \textit{Sporothrix}. Antimicrob Agents Chemother
2008, 52:732–734.
15. Kauffman CA, Hajeh R, Chapman SW, Group MS: Practice guidelines for
the management of patients with sporotrichosis. Clin Infect Dis 2000,
30:684–687.
16. Yamada K, Zaitz C, Framil VM, Muramatu LH: Cutaneous sporotrichosis
treatment with potassium iodide: a 24 year experience in São Paulo
State, Brazil. Revista do Instituto de Medicina Tropical de São Paulo,
53:89–93.
17. de Lima Barros WB, Schubach AO, de-Valconcellos Carvalho De-Oliveira R,
Martins EB, Teixeira JL, Wanke B: Treatment of cutaneous sporotrichosis
with itraconazole—Study of 645 patients. Clin Infect Dis 2011,
52:e200–e206.
18. Rodrigues AM, de Hoog GS, Camargo ZP: Genotyping species of the
\textit{Sporothrix schenckii} complex by PCR-RFLP of calmodulin. Diagn Microbial
Infect Dis 2014, 78:383–387.
19. Fernandes GF, dos Santos PO, Amaral CC, Sasaki AA, Godoy-Martinez P,
Camargo ZP: Characteristics of 151 Brazilian \textit{Sporothrix schenckii} isolates
from 5 different geographic regions of Brazil: A forgotten and
re-emergent pathogen. Open Mycol J 2009, 3:48–58.
20. Espinel-Ingroff A, Chatuev V, Fothergill A, Rinaldi MG: Optimal testing
conditions for determining MICs and Minimum Fungicidal
Concentrations of new and established antifungal agents for uncommon
molds: NCCLS collaborative study. J Clin Microbiol 2002, 40:3776–3781.
21. O’Donnell K, Nienberg H, Koki T, Cigelnik E: A multigene phylogeny of the
Gibberella fujikuroi species complex: Detection of additional
phylogenetically distinct species. Mycosen 2000, 41:61–78.
22. White TJ, Bruns T, Lee S, Taylor J: Amplification and direct sequencing of
fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: A Guide to
Methods and Applications. Edited by Innis M, Gelfand D, Sninsky J, White
T. New York, USA: Academic Press; 1990:315–322.
23. Marimon R, Gené J, Cano J, Guarro J: \textit{Sporothrix luriei}: a rare fungus from
c clinical origin. Med Mycol 2008, 46:621–625.
24. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S: MEGAS:
Molecular Evolutionary Genetics Analysis using maximum likelihood,
evolutionary distance, and maximum parsimony methods. Mol Biol Evol
2011, 28:2731–2739.
25. Felisberto J, Evolution confidence limits on phylogenies: An approach
using the bootstrap. Evolution 1985, 39:783–791.
26. Tamura K: Estimation of the number of nucleotide substitutions when
there are strong transition-transversion and G + C-content biases. Mol
Biol Evol 1992, 9:678–687.
27. Nei M: Molecular evolutionary genetics. New York: Columbia University Press;
1987.
28. Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 2009, 25:141–145.
29. Bandelt HJ, Forster P, Rohl A. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 1999, 16:37–48.
30. de Vienne DM, Giraud T, Martin OC. A congruence index for testing topological similarity between trees. Bioinformatics 2007, 23:319–324.
31. Woolley SM, Posada D, Crandall KA. A comparison of phylogenetic network methods using computer simulation. PLoS ONE 2008, 3:e1913.
32. Oliveira DC, Lopes PGM, Spader TB, Mahl CD, Tronco-Alves GR, Lara VM, Sarto JM, Alves SH. Susceptibilities to antifungal agents of Sporothrix albicans, S. brasiliensis and S. iniae of the S. schenckii complex identified in Brazil. J Clin Microbiol 2011, 49:3047–3049.
33. Brilhante RS, Malaquias AO, Caetano EP, Castelo-Branco DDS, Lima RA, Marques FJ, Silva NF, Alencar LP, Monteiro AJ, Camargo ZP, Bandeira JTD, Cardoso RA, Moreira JA, Silveira CP, Torres-Rodrigues JM, Alvarado-Ramirez E, Murciango-Gonzalo F, Dolande M, Panizo M, Revaka V. MICS and minimum fungicidal concentrations of amphotericin B, itraconazole, posaconazole and terbinafine in Sporothrix schenckii. J Med Microbiol 2009, 58:1607–1610.
34. Rodríguez-Silva F, Capilla J, Mayayo E, Guarro J. Efficacy of posaconazole in murine experimental sporotrichosis. Antimicrob Agents Chemother 2012, 56:2273–2277.
35. Rodríguez-Silva F, Capilla J, Mayayo E, Guarro J. Modest efficacy of voriconazole against murine infections by Sporothrix schenckii and lack of efficacy against Sporothrix brasiliensis. Mycoses 2014, 57:121–124.

Cite this article as: Rodrigues et al.: Genetic diversity and antifungal susceptibility profiles in causative agents of sporotrichosis. BMC Infectious Diseases 2014:14219.