Chapter 2
Sporothrix schenckii Complex: Genetic Polymorphism

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Abstract *Sporothrix schenckii* is an ascomycetous dimorphic fungus that, for over a century, was recognized as the sole agent of sporotrichosis, a subcutaneous mycosis with a worldwide distribution. Based on physiologic and molecular aspects, however, it has been proposed that *S. schenckii* is a complex of distinct species: *S. brasiliensis*, *S. mexicana*, *S. globosa*, *S. schenckii sensu stricto*, *S. luriei*, and *S. pallida* (formerly *S. albicans*). Before the description of these species, several authors had pointed to a high level of genetic diversity among *Sporothrix* spp. strains according to geographic distribution and clinical forms. Phenotypic characterization is usually made through morphology of colony and conidia and biochemical profiles. However, the correlation between molecular data and phenotypic characteristics is fundamental to the identification of the *Sporothrix* complex. Molecular information about the *Sporothrix* species complex is scarce. Until now, *S. brasiliensis* and *S. schenckii s. str.* are the only clinically relevant species of this complex with an elucidated genome sequence, thus limiting molecular knowledge about the cryptic species of this complex, the population structure, and the sexual form of all *S. schenckii* complex species. In this chapter, we focus on the current molecular tools applied to the identification of the *Sporothrix* complex species and on published studies on *Sporothrix* spp. sexuality, and we outline the geographic distribution of *Sporothrix* complex species.

Keywords *Sporothrix schenckii* • Polymorphism • Genetic variability

2.1 Introduction

Sporothrix schenckii is an ascomycetous dimorphic organism (Ascomycota, Pyrenomycetes, Ophiostomatales, Ophiostomataceae) that is found in substrates such as living and decayed vegetation, animal excreta, and soil (Barros et al. 2011;

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Guarro 2012). This fungus is phenotypically characterized by the ability to produce sessile dematiaceous conidia along with hyaline sympodial conidia in its filamentous form, and cigar-shaped yeast-like cells in parasitism or when cultured at 35–37 °C on specific culture media (Barros et al. 2011; Marimon et al. 2007).

For over a century, this species was recognized as the sole agent of sporotrichosis, a subcutaneous mycosis with a worldwide distribution (Lopes-Bezerra et al. 2006). Until 2007, *S. schenckii* was considered a single taxon, although Liu et al. (2003) previously reported the existence of high genetic variation within this species by means of molecular polymorphisms detected by the random amplification of polymorphic DNA (RAPD) technique that were related to the geographical origin of strains and clinical manifestations of sporotrichosis. Later, other studies also reported genetic variation within *S. schenckii* after comparative analysis of clinical strains by RAPD and internal transcriber spacer (ITS) sequencing (Gutierrez-Galhardo et al. 2008; Reis et al. 2009).

Nevertheless, it has been proposed, based on physiologic and molecular aspects, that *S. schenckii*, instead of a single taxon, is a complex of four distinct species: *S. brasiliensis*, *S. mexicana*, *S. globosa*, and *S. schenckii sensu stricto* (Marimon et al. 2007). Later, *S. schenckii* var. *luriei* was considered another species belonging to the *S. schenckii* complex, and was therefore named *S. luriei* (Marimon et al. 2008a). Additionally, other phylogenetic analysis with *S. albicans*, *S. pallida*, and *S. nivea* revealed a significant similarity among them. Therefore, it has been proposed that all these three non-pathogenic species closely related to *S. schenckii* be called *S. pallida* (de Meyer et al. 2008; Romeo et al. 2011). In fact, Romeo et al. (2011) studied the molecular phylogeny and epidemiology of *S. schenckii* complex strains isolated in Italy and demonstrated that 26 environmental strains co-clustered with *S. pallida* and two clinical strains grouped with *S. schenckii s. str*. Furthermore, a recent report showed *S. pallida*, recognized so far as an exclusively environmental species, caused keratitis in a corneal transplant recipient (Morrison et al. 2013). Therefore, *S. schenckii s. str*., *S. brasiliensis*, *S. globosa*, and in minor proportions *S. mexicana*, *S. pallida*, and *S. luriei* are now recognized as agents of sporotrichosis.

Moreover, three other environmental *Sporothrix* species were described using molecular approaches: *S. stylites*, *S. humicola*, and *S. lignivora*. The two first species differ from *S. schenckii* by the sole production of hyaline conidia and consequently shows no darkening of colonies with age. *S. lignivora* has distinctive conidia that do not match in size and shape with other *Sporothrix* or *Ophiostoma* species. Isolates classified as *S. humicola* in this study were previously referred to as environmental *S. schenckii* isolates. In their study, the authors conclude that β-tubulin gene sequence analysis is strongly recommended in taxonomic studies from *Sporothrix* species isolated from the environment (de Meyer et al. 2008). In fact, β-tubulin analysis, together with ITS sequencing, enables the further description of two other environmental *Sporothrix* species: *S. brunneoviolacea* and *S. dimorphospora* (Madrid et al. 2010).

2.2 Differences Among the Species of the *Sporothrix* Complex

Cultures of the members of the *Sporothrix* complex in mycological media such as Sabouraud dextrose agar, potato dextrose agar, or mycobiotic agar yield white filamentous colonies that become brown to black after a few days. Subculturing these colonies in brain heart infusion at 35–37 °C results in white to creamy yeast-like colonies (Barros et al. 2011). Identification of the *Sporothrix* complex is based on the macro and micromorphologies of the mycelial and yeast forms. However, these characteristics do not differentiate the newly described species of the *Sporothrix* complex. In order to physiologically differentiate the species within this complex, other tests such as carbohydrate assimilation (especially sucrose and raffinose), growth rates at 30 °C and 37 °C, as well as production of dematiaceous conidia are necessary (Marimon et al. 2008a). Moreover, the species present variability in several gene sequences, and the partial calmodulin gene sequencing (Marimon et al. 2007) is broadly used to differentiate the species of the *Sporothrix* complex (Fig. 2.1).

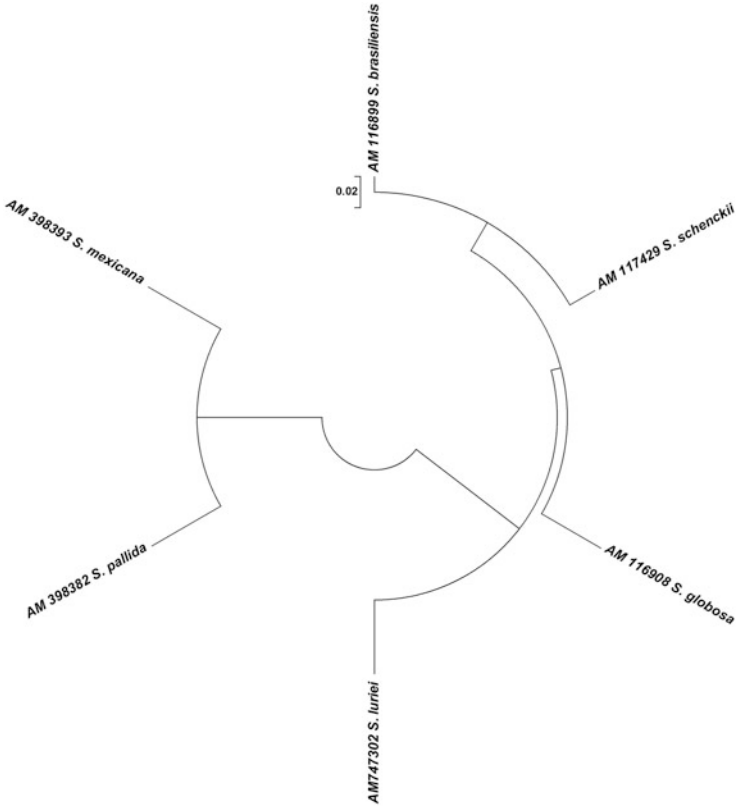

Fig. 2.1 Genetic relationship between the species of the *Sporothrix* complex. Neighbor-Joining tree showing the relatedness of the type strain of each species

Regarding carbohydrate assimilation, *S. schenckii s. str.* and *S. mexicana* are able to assimilate sucrose and raffinose, *S. globosa* and *S. pallida* assimilate only sucrose, and *S. brasiliensis* and *S. luriei* are unable to use any of these two carbohydrates as a sole carbon source for growth. *S. globosa* grows poorly at 37 °C, whereas *S. luriei* grows abundantly at this temperature. Production of dematiaceous conidia is absent in *S. pallida* and *S. luriei*. However, discrepancies between these physiological methods and molecular approaches of identification have been described (Fernandes et al. 2013; Oliveira et al. 2011).

Although geographic limitations are imprecise, epidemiological data indicate that *S. schenckii s. str.* is found predominantly on the American, Asian, and African continents; *S. globosa* has a worldwide distribution with a high frequency in Europe and Asia (Liu et al. 2014; Madrid et al. 2009). *S. brasiliensis* is apparently restricted to Brazil (Marimon et al. 2007; Oliveira et al. 2011; Rodrigues et al. 2013) and *S. mexicana* to Mexican environmental samples (Marimon et al. 2007). However, *S. mexicana* was identified in a case of sporotrichosis in a human patient in Portugal (Dias et al. 2011) and in a re-identification of three clinical isolates maintained in Brazilian fungal collections since 1955 (Rodrigues et al. 2013). *S. luriei* is a very rare pathogen, reported on four human sporotrichosis cases, but isolated only from one case in Africa (Marimon et al. 2008a).

Certain publications have shown that the species of the *Sporothrix* complex differ in virulence and antifungal susceptibility (Arrillaga-Moncrieff et al. 2009; Marimon et al. 2008b). *S. brasiliensis* is described as a highly virulent species, followed by *S. schenckii* and *S. globosa*, which present the lowest virulence among these three species (Arrillaga-Moncrieff et al. 2009). However, the virulence of *S. brasiliensis* does not appear to be related to its antifungal susceptibility, since lower antifungal concentrations are enough to inhibit the growth of this species. On the other hand, *S. mexicana* is highly resistant to most clinically used antifungal drugs, such as fluconazole, itraconazole, voriconazole, ravuconazole, micafungin, and amphotericin B (Marimon et al 2008b).

There are only a few reports regarding the clinical aspects of human sporotrichosis caused by different species of the *Sporothrix* complex. Some publications have suggested that even lesional mechanisms should be related to these different species (Arrillaga-Moncrieff et al. 2009). In general, *S. brasiliensis* is usually associated with unusual clinical manifestations of sporotrichosis such as erythema (Almeida-Paes et al. 2014) or disseminated infection without HIV infection (Orofino-Costa et al. 2013). However, cases of sporotrichosis related to *S. schenckii* (Almeida-Paes et al. 2014) and *S. globosa* (Yu et al. 2013) are closer to the classical description of sporotrichosis cases before reports of the *Sporothrix* complex (Morris-Jones 2002). Therefore, molecular methods that allow rapid identification and differentiation of these closely related fungi are very necessary to the mycology laboratory.

2.3 Sexual Reproduction in the *Sporothrix* Complex: What Is Going On?

Understanding the mechanisms of fungal sexual reproduction is very important since this process strongly influences the level of genetic variability in fungal populations; the study of reproductive biology in *Sporothrix* is crucial because the sexual state of all species of the *S. schenckii* complex is as yet unknown. However, molecular evidence showing that *S. schenckii* undergoes recombination in nature is strong (Mesa-Arango et al. 2002).

Rodrigues et al. (2014a) evaluated the feline host impact on the epidemiology, spatial distribution, prevalence, and genetic diversity of human sporotrichosis and found evidence of recombination in *S. schenckii* but not in *S. brasiliensis*, strongly suggesting that these sibling species follow distinct pathways and strategies during epidemics. The reticulated pattern of *S. schenckii* reported in this study proposes that recombination among genotypes may have contributed to the evolution of divergent strains, and the sexual reproduction in *Sporothrix* is likely to occur in an environmental habitat. However, the feline outbreak genotypes are prevalently clonal, which does not necessarily imply the absence of sex, but does indicate the emergence of a successful genotype.

Molecular analyses from the 18S region of the ribosomal DNA (rDNA) have shown indirect evidence that the *S. schenckii* sexual state was *Ophiostoma stenoceras*. The authors considered that the 18S rDNA gene is highly conserved and may not exhibit sufficient variability to allow a proper distinction between closely related species (Berbee and Taylor 1992). Later, Beer et al. (2003) used ITS sequencing and reported that this anamorph–teleomorph connection was inaccurate. Additionally, morphological and physiological studies showed consistent differences between these two species (Dixon et al. 1991; O'Reilly and Altman 2006). Also, most *Ophiostoma* species have a close association with tree-infesting bark beetles, and some cause tree disease (Suh et al. 2013), and the members of the *Sporothrix* complex are pathogenic fungi that cause human and animal sporotrichosis, with no described cases of plant diseases (Barros et al. 2011). Recently, it has been proposed that the fungus *O. stenoceras* (producing a *Sporothrix* anamorph in culture) is indeed distinct from *S. brasiliensis*, *S. schenckii s. str.*, *S. globosa*, *S. mexicana*, and *S. pallida*, based on calmodulin sequencing (Rodrigues et al. 2013). These results lead us to consider *O. stenoceras* anamorph and *S. schenckii* as distinct species. Meanwhile, molecular studies reinforce that the *Sporothrix* teleomorph belongs to the genus *Ophiostoma*, but that it is different from *O. stenoceras* (Beer et al. 2003; de Meyer et al. 2008; Hintz 1999).

The existence of a sexual state in the *Sporothrix* complex is supported by some studies. In most heterothallic filamentous ascomycete species, the MAT locus bears one of the two idiomorphs, MAT1-1 and MAT1-2, that are required for sexual reproduction and present a low degree of similarity on their gene sequences (Bubnick and Smulian 2007; Casselton 2008). Kano et al. (2013) confirmed the existence of the *MAT1-2* (HMG) gene in *S. globosa* through analysis of its genomic DNA.

The relationships between the *MAT1-2* gene of *S. globosa* and three different species of the genus *Ophiostoma* were also analysed, showing a clear distinction between them, with the four species separated into four clusters. However, a certain degree of homology was noted between the *MAT1-2* gene of *S. globosa* and those of *Ophiostoma* species. The characterization of the partial *MAT1-1* idiomorph (*MAT1-1-1*) of *S. globosa* was performed using the genome walking approach. The *MAT1-1*: *MAT1-2* ratio was also determined on 20 *S. globosa* isolates from clinical cases in Japan. In this study, the authors sequenced *MAT1-1-1* from isolates of *S. schenckii* and *S. globosa*, and the phylogenetic analyses indicated that 92 % of the *MAT1-1-1* sequences between the two species are similar, but they differed from *O. montium*. The intraspecific variation of *MAT1-1-1* was low among isolates of *S. globosa* from different areas in Japan (Kano et al. 2014). Further analysis of the *MAT1-1* gene of members of the *S. schenckii* complex is required, both for its phylogenetic classification as well as for the discovery of teleomorphs of all species in this cryptic complex.

2.4 Genetic Polymorphisms in the *Sporothrix* Complex

Despite the importance of sporotrichosis as a disease with important reported epidemic areas in the last years, just a few studies deal with genetic polymorphisms and genomic architecture of strains of the *Sporothrix* complex. Some publications suggest that molecular polymorphisms of *S. schenckii* can be linked with fungal virulence. A study revealed that a *S. schenckii s. str.* strain isolated from a disseminated cutaneous human sporotrichosis case presented a 10pb deletion in the ribosomal NTS (nontranscribed spacer) region when compared with the control strains obtained from fixed cutaneous sporotrichosis cases (Zhang et al. 2011). Nucleotide polymorphisms are also able to separate environmental and clinical *S. schenckii* strains. Two single-base transitions in the D1–D2 domain of rDNA differentiate strains from these groups (Criseo and Romeo 2010).

Genetic polymorphisms are also likely to be related to antifungal susceptibility in the *Sporothrix* complex. Through a haplotype network approach based on calmodulin and ITS sequences of 22 strains of *S. brasiliensis* and 39 strains of *S. schenckii s. str.*, it has been demonstrated that the epidemic species *S. brasiliensis* has a low genetic diversity and a small variability of susceptibilities to itraconazole and posaconazole. On the other hand, the *S. schenckii s. str.* strains were separated into ten haplotypes, which correlated with the high variability among minimal inhibitory concentrations to the drugs most commonly used in the sporotrichosis treatment (Rodrigues et al. 2014b).

Recently, Sasaki et al. (2014) reported the presence of intra and interspecies polymorphisms in chromosome number and size of 23 strains belonging to the *Sporothrix* complex. In this study, chromosomal polymorphisms and mapping of nine loci (β-tubulin, calmodulin, catalase, chitin synthase 1, ITS, Pho85 cyclin-dependent kinase, protein kinase C Ss-2, G protein a subunit, and topoisomerase II) were studied. The gene hybridization analysis showed that closely related species in

phylogenetic analysis had similar genetic organizations, mostly due to identification of synteny groups in chromosomal bands of similar sizes.

The *Sporothrix* complex is the last clinically relevant group of dimorphic fungi to have an elucidated genome sequence, thus limiting the molecular knowledge about the cryptic species of this complex. Recently, the genome sequence of the *S. schenckii* strain ATCC 58251 was described. The genome size was calculated as approximately 32 Mb with a GC content of 55 %. This genome comprises 8674 protein genes, 111 transfer RNA (tRNA) encoding genes, and 20 rRNA-associated genes (Cuomo et al. 2014). Despite the lack of whole genome information in the *Sporothrix* complex, some genes have been recently described, such as the α-subunit of the endoplasmic reticulum glucosidase II (Robledo-Ortiz et al. 2012), the α1,2-mannosyltransferase (Hernandez-Cervantes et al. 2012), a cytosolic phospholipase A2 (Valentin-Berrios et al. 2009), a guanosine diphosphatase (López-Esparza et al. 2013), an STE20-like protein (Zhang et al. 2013), a histidine kinase associated to dimorphism (Hou et al. 2013), and a calcium/calmodulin kinase gene (Valle-Aviles et al. 2007). These sequences are potential targets for the development of new identification and typing methods to be applied to the species of the *Sporothrix* complex.

2.5 Molecular Identification of *Sporothrix* Species

In recent years, the development of DNA-based methods to identify fungal isolates has led to a decrease in the time-consuming step of morphological identification, while maintaining or improving specificity, accuracy, and sensitivity. Until now, few molecular methods have been applied in the detection of *S. schenckii* DNA from clinical specimens and in the identification of *Sporothrix* spp. in culture (Table 2.1).

As described earlier in this chapter, the most reliable method for the identification of species in the *Sporothrix* complex is partial calmodulin gene sequencing

Table 2.1 Molecular studies for species identification in the *Sporothrix schenckii* complex

Method	Target gene region	Reference
PCR[a] sequencing	Calmodulin, β-tubulin, and chitin synthase genes	Marimon et al. (2007)
PCR sequencing	ITS[c], LSU[d], and β-tubulin	de Meyer et al. (2008)
PCR sequencing	ITS, β-tubulin, chitin synthase genes	Zhang et al. (2011)
PCR fingerprinting	T3B	Oliveira et al. (2012)
PCR sequencing	ITS	Zhou et al. (2014)
RFLP[b]	Calmodulin	Rodrigues et al. (2014c)

[a]Polymerase chain reaction
[b]Restriction Fragment Length Polymorphism
[c]Internal Transcriber Space
[d]Large subunit

(Marimon et al. 2007). After the description of *Sporothrix* complex, one important issue is the search for rapid methods of species identification and typing. Oliveira et al. (2012) reported a polymerase chain reaction (PCR) fingerprinting using the universal primer T3B to distinguish among species of the *Sporothrix* complex. T3B fingerprinting generated clearly distinct banding patterns, allowing the correct identification of all 35 clinical isolates at the species level, which was further confirmed by partial calmodulin gene sequence analyses. Overall, there was a 100 % agreement between the species identification using both genotypic methodologies. These profiles were also able to accurately distinguish the strains misidentified by phenotypic analysis. This proposed identification technique is simple, reliable, faster, less expensive, and requires less technical expertise than sequencing. The computer-scanned PCR profiles generated can form the basis of a computer database that can be used for future identification of atypical or unidentifiable *Sporothrix* isolates. This methodology is supposed to be an ideal routine identification system for clinical mycology laboratories, particularly in those with limited facilities or technical expertise.

In addition, a PCR-restriction fragment length polymorphism (RFLP) targeting the calmodulin gene sequence digested with the restriction enzyme *Hha*I was reported, with five different electrophoretic patterns representing the isolates of *Sporothrix* spp.: *S. brasiliensis*, *S. schenckii s. str.*, *S. globosa*, and *S. luriei*. However, this PCR-RFLP protocol did not permit identification of all species included in this complex, because *S. mexicana* and *S. pallida* yield identical band patterns (Rodrigues et al. 2014c).

In a search for other methodologies with enough discrimination power to differentiate strains of the *Sporothrix* complex, Zhou et al. (2014) suggested that the ITS region analysis could also be applied for identification at the species level. Another study applying the ITS1-5.8S-ITS2 region of the ribosomal DNA reported that this region could be utilized as a broad molecular marker for inter- and intraspecific genetic diversity of the *Histoplasma capsulatum* and *S. schenckii* species complexes and could discriminate *H. capsulatum* or *Sporothrix* isolates according to their geographic distribution and association with environmental sources. However, the authors reported that the ITS regions were able to distinguish neither *H. capsulatum* species nor *Sporothrix* spp. among their respective phylogenetic, biological, and/or taxonomic species complexes (Estrada-Bárcenas et al. 2014).

Only a few molecular tools exist for *Sporothrix* species identification and typing (Oliveira et al. 2012; Zhou et al. 2014; Rodrigues et al. 2014c), and the development of new typing methods are necessary. Further studies for the development of new methodologies for identification and typing of the *Sporothrix* complex should be easy since the genome sequence of *S. schenckii* (ATCC 58251) was recently reported (Cuomo et al. 2014), which will facilitate the study of this and of other species of the *Sporothrix* complex.

2.6 Conclusion and Perspectives

The species of the *Sporothrix* complex present several differences, both at molecular and at phenotypic levels. Rapid and accurate identification to the species level is crucial to correct management of sporotrichosis. Consequently, the correlation between molecular data and phenotypic characteristics is fundamental to the identification of the *Sporothrix* complex. Only a few molecular tools exist for *Sporothrix* species identification and typing. Therefore, better understanding of the strengths and weakness of currently available molecular methodologies would greatly improve the speciation and the intra-variability among the isolates.

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