Asexual Propagation of a Virulent Clone Complex in a Human and Feline Outbreak of Sporotrichosis

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Sporotrichosis is one of the most frequent subcutaneous fungal infections in humans and animals caused by members of the plant-associated, dimorphic genus Sporothrix. Three of the four medically important Sporothrix species found in Brazil have been considered asexual as no sexual stage has ever been reported in Sporothrix schenckii, Sporothrix brasiliensis, or Sporothrix globosa. We have identified the mating type (MAT) loci in the S. schenckii (strain 1099-18/ATCC MYA-4821) and S. brasiliensis (strain 5110/ATCC MYA-4823) genomes by using comparative genomic approaches to determine the mating type ratio in these pathogen populations. Our analysis revealed the presence of a MAT1-1 locus in S. schenckii while a MAT1-2 locus was found in S. brasiliensis representing genomic synteny to other Sordariomycetes. Furthermore, the components of the mitogen-activated protein kinase (MAPK)-pheromone pathway, pheromone processing enzymes, and meiotic regulators have also been identified in the two pathogens, suggesting the potential for sexual reproduction. The ratio of MAT1-1 to MAT1-2 was not significantly different from 1:1 for all three Sporothrix species, but the population of S. brasiliensis in the outbreaks originated from a single mating type. We also explored the population genetic structure of these pathogens using sequence data of two loci to improve our knowledge of the pattern of geographic distribution, genetic variation, and virulence phenotypes. Population genetics data showed significant population differentiation and clonality with a low level of haplotype diversity in S. brasiliensis isolates from different regions of sporotrichosis outbreaks in Brazil. In contrast, S. schenckii isolates demonstrated a high degree of genetic variability without significant geographic differentiation, indicating the presence of recombination. This study demonstrated that two species causing the same disease have contrasting reproductive strategies and genetic variability patterns.

Fungi exhibit a wide diversity of reproductive modes, including sexual, asexual, and parasexual strategies. Sexual reproduction generates genetic variation by meiotic recombination, which may alter virulence, increase fitness in new ecological niches, and purge deleterious mutations from the genome (1, 2). A strictly clonal mode of reproduction may be advantageous where genotypes are adapted to specific hosts and habitats (3). Pathogenic species with unknown sexual cycles usually have phylogenetically close environmental, sexually active counterparts, suggesting that asexual propagation greatly outpaces any outcrossing that might be coupled with the emergence of pathogenic status (4–9).

Members of Sporothrix species are fungal pathogens associated with sporotrichosis, a subcutaneous disease of humans and animals, especially felines (10–12). The infection is mediated by the traumatic inoculation of fungal elements into the cutaneous/subcutaneous tissues or, occasionally, by inhalation followed by pulmonary lesions. The fungi are thermo-dimorphic, living as saprophytes in association with plant debris and decaying organic matter in soil, and exhibiting an invasive yeast-like form in the warm-blooded host (11). Multiple genealogies of chitin synthase, β-tubulin, and calmodulin have shown that several Sporothrix species are involved (13), of which S. schenckii sensu stricto, S. brasiliensis, S. globosa, and S. luecii form a monophyletic clade (14). With the exception of rare infections caused by S. mexicana and S. pallida, sporotrichosis is almost exclusively associated with species in this group (15). Infection experiments in mouse models have shown that clinical isolates of S. brasiliensis, S. schenckii, and S. globosa express different levels of virulence (16, 17). Sporotrichosis is commonly observed in felines (Felis catus), and zoonotic outbreaks have been reported. A 1998–2010 outbreak of 3,244 cases of sporotrichosis in cats, 120 cases in dogs, and 2,200 cases in humans occurred in Brazil, suggesting a sharp increase in human cases correlated with one in domestic cats (12, 18, 19). In the outbreak region of Rio de Janeiro (RJ), S. brasiliensis proved to be overwhelmingly prevalent among cats and humans (20); however, the population structure, epidemiology, and emergence of these pathogens have not been elucidated.
Sporothrix belongs to the order Ophiostomatales (Sordariomycetes, Ascomycota) and is closely related to Ophiostoma, a global genus of plant pathogens distributed by arthropods (15, 21, 22). Sporothrix reproduces asexually, whereas in many Ophiostoma species elaborate sexual fruiting bodies are observed in addition to a Sporothrix-type of sporulation. For many years S. schenckii has been considered to be the asexual stage of Ophiostoma stenoceras (23, 24); however, phylogenetic analysis revealed them to be two distinct species (21, 22, 25). Sexual structures (long-necked perithecia) in Ophiostoma can be easily obtained by in vitro mating on special medium (26). Ophiostoma harbors homothallic species (e.g., Ophiostoma stenoceras and Ophiostoma nigrocarpum) in which isolates are self-fertile, as well as heterothallic species (e.g., Ophiostoma quercus and Ophiostoma novo-ulmi), where two self-sterile individuals are necessary to induce mating (27–30).

Genomic comparisons of various fungi have increased our knowledge of the evolution of reproduction in both homothallic and heterothallic species (31–33). The mating process and sexual reproduction in Ascomycota are controlled by the mating type genes (MAT) which encode transcription factors that are essential for mating (34, 35). However, little is known at the molecular level of the sexual cycle of Ophiostomatales; MAT loci and flanking genes have been identified only in Ophiostoma ulmi, O. novo-ulmi, Ophiostoma hinal-ulmi, and O. quercus (27, 36–38). Recently the MAT genes have been characterized in six Grommannia species, as well as in Ophiostoma montium, and unequal recombination has been suggested to account for the presence of a truncated MAT1-1 in isolates of the opposite mating type (39). Although sexual reproduction has never been observed, the sexual state of Sporothrix species is predicted to be Ophiostoma-like based on the observation that Sporothrix is phylogenetically nested within Ophiostoma (21, 22).

The present study aimed to identify the MAT locus and sex-related genes in the genomes of S. schenckii (strain 1099-18/ATCC MYA-4821/CBS 132984) and S. brasiliensis (strain 5110/ATCC MYA-4823/CBS 132021) using comparative genomic analysis. Furthermore, this study assessed the distribution of sexual idiomorphs MAT1-1 and MAT1-2 in isolates of the S. schenckii relatives and the influence of breeding strategies in the genetic structure of the disease, especially in the recent outbreaks in Brazil. For this, we used both comparative genomic and phylogenomics approaches to identify the role of sexual recombination in the genetic structure of this epidemic. We aim to answer three specific questions: (i) Do the Sporothrix genomes demonstrate evidence of sex? (ii) What are the population structures with regard to their mating type/sex ratio? (iii) What are the genetic variations in populations of S. schenckii and S. brasiliensis? Together, the data reveal differences in population structures and breeding strategies of S. schenckii and S. brasiliensis.

**MATERIALS AND METHODS**

**Mating type locus characterization and evolution.** The mating type loci of S. schenckii and S. brasiliensis were identified using the MAT1-1,1-MAT1-1,2, MAT1-1,3, and MAT1-2,1-2 genes of Neurospora crassa and Magnaporthe grisea as queries. Searches were performed using tblAST against the assembled scaffolds from the S. schenckii (strain 1099-18/ATCC MYA-4821/CBS 132984; GenBank accession number AXCR00000000) and S. brasiliensis genomes (strain 5110/ATCC MYA-4823/CBS 132021; accession number AWTV00000000). The boundaries of both MAT1-1 and MAT1-2 loci within a 20-kb scaffold fragment containing both mating types were determined by reciprocal alignment using ClustalW (41) implemented in the BioEdit software (42). We also characterized the MAT1-2 idiomorph of S. globoza, another human pathogen, using long-range PCR amplification with a primer-walking sequencing approach (39).

**Identification of sex-related genes in S. schenckii and S. brasiliensis genomes.** The presence of 108 genes involved in the mating process, mating signaling, fruiting body development, karyogamy, and meiosis was assessed in the S. schenckii and S. brasiliensis genomes. The sex-related predicted proteins previously characterized experimentally in model organisms such as Aspergillus nidulans and N. crassa were used as queries to identify putative orthologs in the predicted proteomes from two analyzed species (43). Homology was inferred via BLASTp search, and putative orthologs were considered with a minimum query/subject coverage of 60% and a minimum positive of 50% using an E value cutoff of e<–13.

**Long-range PCR and primer walking.** Primers ER (GCCACGCTGTTTACAAACTA) and SchMGI (GCTCTCCTTTACAGCTTGTT) targeting the SLA and the MAT1-2 genes of the MAT locus were designed for long-range PCR in Sporothrix globerus. Long-range PCR amplifications of DNA were carried out in 50 μl using an Applied Biosystems 2720 thermal cycler (Life Technologies Corp., Carlsbad, CA, USA). Reaction mixtures contained 100 ng of DNA, 1× PCR buffer, 200 μM each dNTP, 1 μM each primer (Eurofins MWG Operon, Huntsville, AL, USA), 3% dimethyl sulfoxide (DMSO), and 2 U of Phusion DNA polymerase (Finzymes, BioLabs, New England, USA). The PCR amplifications were performed for 30 s at 98°C, followed by 35 cycles of 10 s at 98°C, 30 s at 60°C, and 4 min at 72°C, with a final extension at 72°C for 10 min. Sequencing reactions with primer walking were performed at the Centre de recherche du CHUQ (CHUL), Québec, Canada. Primers for sequencing are listed in Table S1 in the supplemental material. Sequence reads after primer walking were assembled using the Staden package (44) and Geneious Pro (Biomatters, Auckland, New Zealand), and sequences were compared with genes present in GenBank using BLASTx and BLASTn. The assembled sequences were submitted to FGENSEH+ in Softberry (Softberry, Inc., Mount Kisco, NY) for gene prediction and to determine the location of the coding/noncoding regions. The nucleotide sequences of the MAT idiomorphs were compared using dot plot (matrix) analyses implemented in Geneious, version 5. The sequences of the MAT loci were also compared across different species using BLASTx in the Artemis comparison tool (http://www.webact.org/WEBACT/home). The amino acid sequences encoded by the MAT1-1 α-box and the MAT1-2 high-mobility group (HMG) domain in Sporothrix species were aligned to sequences from other ascomycetes using ClustalW (41) implemented in Geneious. Sequences from Verticillium dahliae and Neurospora tetrasperma (Sordariomycetes) were selected as outgroups. Phylogenetic analysis was carried out with the neighbor-joining (NJ) method in MEGA, version 5 (45), and the maximum-likelihood (ML) method in PhyML (46) available at http://www.phylogeny.fr/version2_cgi/index.cgi.

**Quantitative expression of MAT genes in yeast and mycelial forms of dimorphic S. schenckii and S. brasiliensis.** We used reverse-transcription (RT) real-time quantitative PCR (qPCR) to assess the levels of expression of MAT1-1,1-MAT1-1,2-MAT1-1,3, and truncated MAT1-1 genes. S. schenckii 1099-18 and S. brasiliensis 5110 isolates were cultured in both yeast and mycelium forms. Mycelia were cultured in Sabouraud broth for 10 days at 25°C under agitation. Yeast cells were obtained after transfer of 10⁶ conidia per ml in brain heart infusion (BHI) broth for 10 days at 37°C under agitation. Cells were then collected by centrifugation and plated on BHI slants to stabilize the yeast phase. Yeast cells were then grown again in BHI broth for 10 days at 37°C under agitation. Cells were collected by centrifugation (5,000 × g for 10 min) and washed three times with 1× phosphate-buffered saline (PBS), and RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. DNA was removed using RNA-free DNase I (Promega), followed by ethanol precipitation. RNA samples were then used for real-time RT-qPCR. Two micrograms of RNA was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-qPCR (Invitrogen). The RNA samples were reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-qPCR (Invitrogen).
transcribed (Superscript III; Invitrogen) using an oligo(dt)12-18 primer and then subjected to real-time RT-PCR. Amplification assays were performed in a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA), using a 10-μl reaction volume containing 0.2 μM (each) primers (see Table S1 in the supplemental material), 5 μl of 2× SYBR green PCR master mix, 0.2 μl of cDNA template, and H₂O. After initial denaturation at 95°C for 20 s, amplifications were performed for 40 cycles at 95°C for 3 s and 60°C for 20 s. All reactions were carried out in triplicate. In order to confirm the amplification specificity, PCR products were subjected to a melting-curve analysis. The comparative crossing threshold (Cₜ) method, employing the constitutive S. chenckii and S. brasiliensis L34 ribosomal gene for normalization, was used to evaluate the expression value for each gene of interest. Primers targeting the five genes at the opposite MAT idiomorphs were designed using Primer, version 3 (http://primer3.wi.mit.edu/).

DNA extraction and MAT locus distribution in the Sporothrix complex. Genomic DNA was extracted and purified from mycelial colonies using a Fast DNA kit protocol (MP Biomedicals, Vista, CA, USA). Mycelial cells were homogenized three times with a Precellys 24 instrument (Bertin, Montigny le Bretonneux, France). DNA integrity was confirmed by 9.8% agarose gel electrophoresis with ethidium bromide (0.5 μg/ml) staining and quantified with a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA). For PCR amplification, 20 to 50 ng of genomic DNA from each isolate (see Table S2 in the supplemental material) was used for PCR amplifications. Primers were designed to target regions inside the MAT1-1-1 (Mat1_1_1F, AAGCACG CGATTTCATCATT; Mat1_1_1R, CACCAAGGAGCATCTCAGT) and MAT1-1-2-1 (Mat1_2F, GATCTCAACGGCCATCTTGT; Mat1_2R, GCT ACATACTTGGCCCTGA) open reading frames (ORFs). PCR amplifications were done in a Mastercycler Pro thermocycler (Eppendorf) and using 2× PCR master mix (Promega) as recommended by the manufacturer. The annealing temperature for both regions was 60°C. PCRs were performed separately for each idiomorph, and amplicons were loaded onto a 0.8% agarose gel and analyzed by electrophoresis. Amplified bands corresponding to each sexual idiomorph of each isolate were assigned. Mating type distributions were tested for deviation from the expected ratio of 1:1 using a chi-square test in Microsoft Excel.

Population genetic studies between S. chenckii and S. brasiliensis. Population studies were done based on the large data sets (n = 133) of calmodulin and EF1α sequences from S. chenckii and S. brasiliensis species (see Table S2 in the supplemental material). Sequences from both loci were aligned using ClustalW (41) and manually adjusted. Nucleotide substitution models were selected using lower Bayesian information criteria (BIC) scores based on ML values computed automatically in a given tree topology implemented in MEGA, version 5, software (45). The evolutionary distances used for phylogenetic inferences were determined using the Kimura two-parameter method (47), and the rate variation among sites was modeled with a gamma distribution (shape parameter, 1). Phylogenetic analyses were carried out using maximum-likelihood and Bayesian inferences implemented in the MEGA, version 5 (45), and MrBayes, version 3.1, software programs (48), respectively. For maximum-likelihood analysis, 1,000 bootstrap replicates were used to estimate confidence values for individual clades. For Bayesian analysis using the Markov chain Monte Carlo (MCMC) method, two independent analyses of four chains each as a default were initiated from a random tree and processed for 1 million generations; sample trees were retrieved every 100 generations. Log-likelihood scores were plotted against the generation number in order to evaluate convergence; samples collected prior to burn-in (25%) were ignored. The remaining samples were used to determine the distribution of posterior probability values (49). Trees were visualized using FigTree software, version 1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Polymorphism comparisons between and within S. chenckii and S. brasiliensis populations were measured using DnaSP, version 5 (50), and the number of variable sites (S), total number of mutations (Eta; H), nucleotide diversity per site (π), number of haplotypes (h), haplotype diversity (hₒ), average number of nucleotide differences (k), mean evolutionary diversity for the entire population (Mₑ), and substitutions per site (Sₑ) were estimated for cal and efl loci. In order to evaluate the reproductive modes of both S. chenckii and S. brasiliensis, we performed recombination analysis using different approaches. Recombination events were inferred using the split decomposition method via the LogDet algorithm and the pairwise homoplasy index (PHI) test for phylogenetic heterogeneity, both implemented in the SplitsTree, version 4.1 (51).

Haplotypic networks were built to visualize differences and diversity among S. chenckii and S. brasiliensis populations in Brazil. The distribution and diversity of haplotypes for cal plus efl were estimated using the software DnaSP, version 5, and used for input in Network, version 4.610, software (Fluxus Technology, Clare, Suffolk, England). Gaps and missing data were excluded in the analysis. Median-joining networks (52) for the concatenated data set were obtained and visualized using the software Network, version 4.610.

Genetic differentiation among local populations of S. brasiliensis (four) and S. chenckii (seven) was calculated using the analysis of molecular variance (AMOVA) with Arlequin, version 3.11 (53). Fₛₛ is analogous to the fixation index, Fₛ, and incorporates sequence divergence among haplotypes in addition to changes in haplotype frequency to assess genetic differentiation among populations (54). The statistical significance of the PHI statistic was tested based on 1,000 permutations (default settings).

RESULTS

Genomic characterization of mating type locus. The whole-genome sequences of S. chenckii and S. brasiliensis isolates obtained, respectively, from human and cat infections were generated using Roche’s 454 pyrosequencing (40). Genome searches using the MAT1-1-1, MAT1-1-2, MAT1-1-3, and MAT1-2-1 genes of N. crassa and Magnaporthe grisea as queries revealed the presence of a single MAT-1 locus in the S. chenckii genome (GenBank accession number JX105435) and a MAT-1 locus in the S. brasiliensis genome (GenBank accession number JX105956), indicating that the two Sporothrix species are heterothallic (Fig. 1A). The MAT1-2 locus of S. globosa isolate KMU 3314 was characterized using a primer-walking approach (GenBank accession number KF021931). The gene order and orientation flanking the Sporothrix MAT loci were syntenic in all species investigated (see Fig. S1 in the supplemental material). The MAT1-1 locus of S. chenckii contains a region of 4,706 bp that is absent in the S. brasiliensis genome. In contrast, the MAT1-2 locus of S. brasiliensis is composed of 3,133 bp corresponding to a nonhomologous region in the S. chenckii scaffold (Fig. 1B).

Similar to other representatives in Sordariomycetes, the S. chenckii and S. brasiliensis MAT locus is flanked by SLA2, which encodes the cytoskeleton assembly control protein (ORFs SP505860 and SPBR04592), cytochrome c oxidase subunit COX13 (ORFs SPBR05223 and SPKS06524), and DNA lyase APN2 (ORFs SPKS05918 and SPBR04646) (55) (Fig. 2). The deduced amino acid sequences of SLA2 and APN2 shared, respectively, 89% and 62% identity with those of Grosmannia clavigera, a tree pathogen in the Ophiostomatales (39). Three open reading frames (ORFs) were predicted in the MAT1-1 idiomorph of the S. chenckii genome: MAT1-1-1, MAT1-1-2, and MAT1-1-3, encoding proteins of 893, 362, and 191 amino acids (aa), respectively (Fig. 1A). The first protein had the α-box domain and was homologous (50% similarity) to the MAT1-1-1 of Ophiostoma novo-ulmi subsp. novo-ulmi (GenBank accession number ACZ53927.1). The second protein had an invariant PF domain and was 35% similar to MAT1-1-2 of O. montium (GenBank accession number
The third ORF encoded an HMG box domain protein sharing 65% similarity to the MAT1-1-3 of O. montium (GenBank accession number AGH03256.1). Two ORFs were predicted in the MAT1-2 idiomorphs of S. brasiliensis and S. globosa. The translated protein bearing the HMG domain (272 aa from S. brasiliensis) shared 90% similarity to that of S. globosa and was homologous to the MAT1-2-1 genes of other Sordariomycetes (see Fig. S1 in the supplemental material). tBLAST searches using the H9251-box domain containing genes from S. schenckii as queries did not return any significant matches in the S. brasiliensis genome, confirming the absence of the MAT1-1-1 gene.

The 734-amino-acid hypothetical proteins upstream from the MAT1-2-1 genes in both S. globosa and S. brasiliensis had no significant similarity to any genes in the NCBI sequence database. Surprisingly this putative protein in S. brasiliensis (GenBank accession number JX101596) and S. globosa (GenBank accession number KF021931) was partially homologous (>60% similarity in amino acids) to the MAT1-1-1 gene in the MAT1-1 of S. schenckii but shorter in length and with no introns (see Fig. S2 in the supplemental material). Sequence comparison also revealed an α-box domain truncation/deletion at the N terminus of the putative protein. The start codon, however, was present in the putative protein coding sequence (called truncated MAT1-1-1), followed by fragments that are not homologous to the MAT1-1-1 in the MAT1-1 idiomorph (see Fig. S2 in the supplemental material). This 179-bp N-terminal sequence was used to search the genome, as well as the NCBI database, but no significant match was returned. Phylogenetic analysis of a 225-amino-acid stretch from the HMG domain corresponding to the MAT1-2-1 gene grouped S. brasiliensis and S. globosa in a strongly supported monophyletic clade, and they are divergent from representatives of Grosmannia and Ophiostoma (see Fig. S3). Representatives of ophiostomatoid fungi clustered with members of Neurospora and other Sordariales, with strong likelihood support (data not shown).
shown). This suggested that the human/animal pathogens might have evolved from the plant and forest pathogens within the Ophiostomatales.

The mRNA levels of the MAT1-1-1, MAT1-1-2, MAT1-1-3, MAT-1-2-1, and the truncated MAT1-1-1 genes were quantified by qPCR analysis of S. schenckii and S. brasiliensis in both mycelial and yeast stages. The genes MAT1-1-1, MAT1-1-2, and MAT1-1-3 were expressed in S. schenckii 1099-18 while MAT-1-2-1 was expressed in S. brasiliensis 5110 (Fig. 3). Surprisingly, mRNA from the truncated MAT1-1-1 gene of S. brasiliensis was detected, suggesting that this ORF is transcribed despite the absence of the α-box domain.

Identification of sex-related genes in Sporothrix genomes. We evaluated the presence or absence of 108 genes involved in the mating process, mating signaling, fruiting body development, karyogamy, and meiosis known from a wide range of fungi in the S. schenckii and S. brasiliensis genomes. Reciprocal best BLAST hits revealed the presence of 106 putative orthologs involved in sexual development in the Sporothrix genomes (see Table S3 in the supplemental material). In addition to the mating type idiomorphs, pheromone precursor and pheromone receptor genes, pheromone degrading enzymes, and meiotic genes were detected in the two Sporothrix genomes. The genes involved in the classical mating mitogen-activated protein kinase (MAPK) signaling cascade were also found highly conserved in these two pathogens. The presence of the MAP kinases, pheromone G-coupled protein subunits, and the transcriptional activator containing the homeodomain DNA-binding factor ste12 could also be recognized. Additionally, the majority of genes involved in fruiting body development were also found in both Sporothrix genomes. The only two genes not identified in the Sporothrix genomes related to fruiting body development were rhamnogalacuronase B and muta-
The Sporothrix species harbor the majority of genes involved in pheromone activation, karyogamy, and meiosis in their genomes, thus indicating that both *S. schenckii* and *S. brasiliensis* possess the genomic apparatus to complete a sexual cycle.

**Mating type distribution.** Primers targeting *MAT1-1* and *MAT1-2* loci were designed to investigate the mating type distribution in the *Sporothrix* populations in Brazil. A total of 193 isolates comprising *S. schenckii* (*n* = 99), *S. brasiliensis* (*n* = 90), and *S. globosa* (*n* = 4) were examined, revealing the presence of a single copy of the *MAT1-1* or *MAT1-2* locus in their genomes (single amplified band), suggesting that they are all heterothallic (see Fig. S4 and Table S2 in the supplemental material). All three *Sporothrix* species have approximately equal ratios of the *MAT1-1* and the *MAT1-2* idiomorphs, suggesting the presence of random mating within each species (chi-square test, *P* values of >0.05) (Table 1). Analysis of the mating type frequencies in populations of *S. schenckii* and *S. brasiliensis* also indicated a 1:1 ratio of both mating types in most states of Brazil (Fig. 4). However, in Rio Grande do Sul (RS) and Minas Gerais (MG), the frequency of *MAT1-1* isolates was significantly higher in *S. brasiliensis* (*P* < 0.001) (Fig. 4), while *MAT1-2* isolates were dominant in Rio de Janeiro (RJ) (*P* < 0.001). In contrast, *S. schenckii* appeared to have a 1:1 ratio of both mating types in the states of São Paulo (SP), Paraná (PR), and Goiás (GO) (*P* > 0.05) (Fig. 4).

**Population genetics and recombination analysis.** All *S. brasili-

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**TABLE 1** Mating type distribution of *Sporothrix* species

| Species        | MAT1-1 | MAT1-2 | Chi-square value |
|----------------|--------|--------|------------------|
| *S. schenckii* | 45     | 54     | 0.366            |
| *S. brasiliensis* | 37     | 53     | 0.092            |
| *S. globosa*    | 2      | 2      | 1.000            |
| Total          | 84     | 109    | 0.072            |

* Chi-square test was used to test mating type distributions for deviations from equal ratios, suggesting random mating. All of the values were nonsignificant.

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**FIG 4** Geographic and mating type distribution of *S. schenckii* and *S. brasiliensis* isolates along the Brazilian territory discriminated by states. Mating type distributions (*MAT1-1* and *MAT1-2*) were tested for deviation from the expected ratios of 1:1 using a chi-square test, and *P* values were added to graphs in order to infer statistical support. AC, Acre; AL, Alagoas; AM, Amazonas; AP, Amapá; BA, Bahia; MA, Maranhão; MS, Mato Grosso do Sul; MT, Mato Grosso; PA, Pará; PI, Piauí; RN, Rio Grande do Norte; RO, Rondonia; RR, Roraima; SC, Santa Catarina; SE, Sergipe; TO, Tocantins.
schenckii isolates formed a single, well-supported, monophyletic group based on the phylogenetic analyses of partial sequences of calmodulin (cal) and translation elongation factor 1-alpha (efl) (see Fig. S5 in the supplemental material). S. schenckii isolates also formed a monophyletic group, but the population was divided into multiple subclades, with low to moderate statistical support (see Fig. S5). Therefore, the S. brasiliensis population is dominated by a single genotype, while the S. schenckii population is composed of multiple genetic lineages. Polymorphism analysis also revealed large variations between the genetic profiles of these two species. We evaluated the polymorphisms in the cal and efl loci considering both species separately. The cal locus was more polymorphic than that of efl in all aspects analyzed. The measurements of the number of variable sites (S), total number of mutations (Ht), nucleotide diversity per site (θ), number of haplotypes (h), haplotype diversity (θπ), average number of nucleotide differences (k), and mean evolutionary diversity for the entire population (MDa) clearly show that S. schenckii is highly diverse compared to S. brasiliensis, and the pattern is in agreement with the phylogenetic analysis (Table 2).

In order to evaluate reproductive isolation within S. schenckii and S. brasiliensis, we performed various recombination analyses. The split decomposition method generated by the LogDet algorithm showed recombination networks linking isolates from S. schenckii in the São Paulo, Goiás, and Paraná states of Brazil (Fig. 5A to C). A PHI test also detected recombination (P = 0.018) between isolates of S. schenckii in the São Paulo state. On the other hand, we have not identified any recombination network in S. brasiliensis isolates, suggesting a clonal reproductive mode there (Fig. 5D to F), which supported the uneven distribution of different mating types along the Brazilian territory.

Median-joining networks were generated using the concatenated sequences of cal and efl loci for S. schenckii and S. brasiliensis populations independently (Fig. 6). The small number of haplotypes in S. brasiliensis suggests low genetic diversity, while in S. schenckii an increased haplotype diversity was observed. The haplotype networks show that the S. schenckii population presents high genetic variability and recombination of S. schenckii in Brazil, as deduced by the presence of several medial vectors linking different haplotypes (Fig. 6A). No strong geographic differentiation structure was observed, and mixed mating types were observed in the S. schenckii population (Fig. 6A and C). On the other hand, strong geography-based structuring was observed in the S. brasiliensis population (Fig. 6B). Four major populations were observed, each geographically restricted and with a predominant mating type, suggesting clonal expansions (Fig. 6D). The RS population composed of haplotypes H4 and H9 predominantly had the mating type MAT1-1, while the in RJ population composed of the haplotypes H2, H7, H8, and H10, the MAT1-2 idiomorph was predominant (Fig. 6D).

AMOVA was used to estimate population differentiation in each of the two Sporothrix species. Only 27.56% of the genetic variation in S. schenckii was attributed to differences between populations; in contrast, between-population variation in S. brasiliensis was more than double that observed in S. schenckii (66.4%), taking into account that the samples were collected on a smaller geographic scale (see Table S4 in the supplemental material).

**DISCUSSION**

Sexual reproduction in fungi promotes recombination and is thought to be more efficient in removing deleterious mutations (1). However, many pathogenic fungi could maintain the ability to carry out the sexual cycle and recombination, yet they drive clonal expansion after adaption to newly acquired environmental and host conditions. As a result, it is not uncommon that the core genes required for sexual development are found in the genomes of limiting-sex fungi (32, 55). In asexual pathogenic fungi such as Paracoccidioides brasiliensis, Paracoccidioides lutzii, and A. fumigatus, the sex-related genes were identified in their genomes prior to identification of sexual morphology (56, 57). In our investigation, different pathways of mating processes present in the genomic architecture of sexual fungi, such as the mating signaling cascade, fruiting body development, karyogamy, and meiosis, were detected in S. schenckii and S. brasiliensis genomes, providing molecular evidence to support the potential to perform mating/sexual recombination in these species (see Table S3 in the supplemental material). The identification of a single mating type locus bearing either an HMG or an α-box indicates that these species are heterothallic (Fig. 1). The presumed heterothallism in these species is also confirmed by the amplification of a single mating type in all 196 isolates tested. The general SLA-MAT-APN pattern, as well as the synteny and orientation of the MAT1-1-1, MAT1-1-2, and MAT1-1-3 genes on the MAT1-1 idiomorph, is consistent with other representatives of Grosmannia and Ophiostoma in the Ophiostomatales (39, 58). This pattern suggests a shared common heterothallic ancestor among members of the Ophiostomatales. However, apart from heterothallic species (e.g., O. querus and O. novo-ulmi), Ophiostoma also harbors homothallic species (e.g., O. stenoceras and O. nigrocarpum), indicating different evolutionary strategies for breeding (28, 29). Also we detected by quantitative RT-PCR the expression of the mating type genes in a sex-type-specific manner, indicating heterothallism and functional activation of the MAT locus (Fig. 3).

The occurrence of truncated MAT1-1-1 genes in the MAT1-2 idiomorphs in Sporothrix provides strong evidence for unequal recombination at the MAT locus in an ancestor of the ophiostomatoid fungi. Truncated MAT1-1-1 genes were already reported in various representatives of Grosmannia and Ophiostoma (39), as well as other ascomycetes (27, 59, 60). The loss of a functional domain (in this case an α-box) with mutations and the high
level of divergence between the truncated MAT1-1-1 and the full-length MAT1-1-1 suggest that these truncated MAT genes might have drifted under relaxed selective pressure (39) or might have been inactivated due to signal interference if both the HMG and \(\alpha\)-box domains were present. Our data also demonstrated the variation in the rate of truncated MAT1-1-1 degeneration among Grosmannia, Ophiostoma, and Sporothrix. We detected expression of the truncated MAT1-1-1 in S. brasiliensis, and hence this gene may have gained a new function apart from mating in Sporothrix.

Our investigation presented challenging questions on the biology of Sporothrix. The S. schenckii species complex has been considered asexual because of a lack of morphological evidence on sexual development. However, genomic evidence indicated that S. schenckii, S. brasiliensis, and S. globosa are heterothallic, and they may retain the ability to reproduce sexually, probably in the same fashion as other sexual fungi in Ophiostomatales (61, 62).

Sexual recombination may drive the formation of a novel, successful genotype that can replicate in mass numbers through asexual propagation (1, 63). Sexual reproduction increases the rate of adaptation to new environments by increasing the mutation rates in a fungal offspring. The reduction of sexual status and of recombination rates between species is correlated with a limited potential to disperse within different environments, but once a species is well adapted to an environment, asexual reproduction may preserve a successful genotype (3, 6). The extremely skewed ratios observed between mating types in Rio Grande do Sul, Minas Gerais, and, especially, Rio de Janeiro suggest the presence of a local clonal lineage or a recent introduction of the opposite mating
type of **S. brasiliensis** during the outbreaks. Local differences may exist in virulence-associated genes that may show chromosomal linkage to the **MAT** locus. Due to the presence of feline sporotrichosis outbreaks in the states of Rio de Janeiro and Rio Grande do Sul, we hypothesize that this deviation is related to the form of transmission, cat to cat and cat to human, which favors the emergence of a clonal genotype in particular. The uneven mating type ratio in these states also indicates clonal expansion of **S. brasiliensis**.

We hypothesize that the origin of cat/human sporotrichosis was the result of a recent spread of **S. brasiliensis**, with increased virulence, into new areas of Brazil where the fungus encountered susceptible cat hosts. Infection assays using mouse models revealed a higher virulence of **S. brasiliensis** than of **S. schenckii**, supporting the spread of virulent clone complexes in sporotrichosis epidemics (17, 64). According to the phylogenetic trees (see Fig. S5 in the supplemental material), a monophyletic branch of a single mating type was observed in the **ef1** phylogeny that was composed of isolates from Rio Grande do Sul and, thus, suggested the dispersal of a new genetic variant of **S. brasiliensis**. A pronounced geography-based population structure was observed in **S. brasiliensis** (Fig. 6B), and four major geographically restricted populations were detected. Each **S. brasiliensis** population harbored a single mating type, suggesting clonal expansions of specific genotypes, even though both mating types can be found in the same clinical and natural environments, making sexual reproduction possible (65).

Two species of **Sporothrix** differ in their major modes of reproduction, as indicated from the incongruent population structure patterns and contrasting genetic signatures. **S. schenckii** was a highly diverse population compared to **S. brasiliensis** based on polymorphism data of **cal** and **ef1** loci, in agreement with the phylogenies and haplotype networks. Recombination analysis by split decomposition shows networks linking isolates of **S. schenckii** in the São Paulo, Goiás, and Paraná states of Brazil (Fig. 5A to C), which is in agreement with sexual recombination. These findings
strongly suggest other forms of acquisition of the disease, such as those based on environmental contamination with soil and decaying wood. In contrast, the lack of recombination networks and the uneven distribution of opposite mating types among various states suggest a clonal reproductive mode in Sporotrichosis is an important subcutaneous mycosis affecting humans and animals and mainly occurs in the form of epidemics, which may be very large. Areas of endemicity with outbreaks of thousands of cases have been recognized in South Africa, China, and Brazil (Y. Zhang, F. Hagen, B. Stielow. A. M. Rodrigues, K. Samerpitak, Z. Xun, P. Feng, L. Yang, M. Chen, S. Deng, W. Liao, R. Y. Li, F. Li, J. F. Meis, J. Guarro, and G. S. de Hoog, unpublished data). Our study suggests that the high levels of effective clonality and endemicity found in Sporothrichosis may have more to do with MAT expansion. The fungus may have specific host interactions while Sporothrix may generate population genetic diversity through sexual recombination. Interestingly, these two fungi cause the same disease while presenting different population structures. Sexual reproduction enhances the rate of adaptation to new environments by increasing mutation rates in fungal offspring. Limiting sex and recombination between species are correlated with a reduced potential to disperse within different environments. For example, restricted dispersal and adaptation to particular niches were associated with asexuality in the human and animal pathogen Talaromyces (Penicillium) marneffei (7, 67). Similarly, clonally restricted Australian and Papua New Guinean populations of the basidiomycetous yeast Cryptococcus gattii type VGI were detected in regions of cryptococcosis endemicity (68). In addition, a clonal population structure of the thermophilic fungus Paracoccidioides brasiliensis PS3 was suggested (69) and supported by recent dispersion from the Brazilian shield to Colombia (70). The same hypothesis might apply in virulent clonal lineages of S. brasiliensis in cats in geographically restricted parts of Brazil, as deeply explored in this work.

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