


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 gene 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. luriei* 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.

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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 himal-ulmi*, and *O. quercus* (27, 36–38). Recently the *MAT* genes have been characterized in six *Grosmannia* species, as well as in *Ophiostoma montium*, and unequal recombination has been suggested to account for the presence of a truncated *MAT1-1-1* 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* genes of *Neurospora crassa* and *Magnaporthe grisea* as queries. Sequences were searched (tBLAST) against the assembled scaffolds from the *S. schenckii* (strain 1099-18/ATCC MYA-4821/CBS 132984; GenBank accession number [AXCR000000000](#)) and *S. brasiliensis* genomes (strain 5110/ATCC MYA-4823/CBS 132021; accession number [AWTV000000000](#)) (40). 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) im-

plemented in the BioEdit software (42). We also characterized the *MAT1-2* idiomorph of *S. globosa*, 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 $1e^{-13}$.

Long-range PCR and primer walking. Primers ER (GCCACGTCGT TCAACAATA) and ScHMGf (GCCTCCCTTACAGCTCTGTG) targeting the *SLA* and the *MAT1-2-1* genes of the *MAT* locus were designed for long-range PCR in *Sporothrix globosa*. Long-range PCR amplifications of DNA were carried out in 50 μ l using an Applied BioSystems 2720 thermal cycler (Life Technologies Corp., Carlsbad, CA, USA). Reaction mixtures contained 100 ng of DNA, $1\times$ PCR buffer, 200 μ M each deoxynucleoside triphosphate (dNTP), 1 μ M each primer (Eurofins MWG Operon, Huntsville, AL, USA), 3% dimethyl sulfoxide (DMSO), and 2 U of Phusion DNA polymerase (Finnzymes, 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 FGENESH+ 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 BLASTn 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*, *MAT1-2-1*, and truncated *MAT1-1-1* 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 10^6 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 medium 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 \times g for 10 min) and washed three times with $1\times$ phosphate-buffered saline (PBS), and RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. DNA was removed using RNase-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 (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- μ l reaction volume containing 0.2 μ M (each) primers (see Table S1 in the supplemental material), 5 μ l of 2 \times SYBR green PCR master mix, 0.2 μ l 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 (C_T) method, employing the constitutive *S. schenckii* 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 0.8% agarose gel electrophoresis with ethidium bromide (0.5 μ l/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 CCAGTTTCATTCT; Mat1_1_1R, CACCAACGAGCATCTCATGT) and *MAT-1-2-1* (Mat1_2F, GATCTCAACGGCCATCTTGT; Mat1_2R, GCT ACATACTTCCGCCCTGA) open reading frames (ORFs). PCR amplifications were done in a Mastercycler Pro thermocycler (Eppendorf) and using 2 \times PCR master mix (Promega) as recommended by the manufacturers. 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. schenckii* and *S. brasiliensis*. Population studies were done based on the large data sets ($n = 133$) of calmodulin and EF1 α sequences from *S. schenckii* 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. schenckii* 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 (h_d), average number of nucleotide differences (k), mean evolutionary diversity for the entire population (M_{cd}), and substitutions per site (S_{ps}) were estimated for *cal* and *ef1* loci. In order to evaluate the reproductive modes of both *S. schenckii* 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. schenckii* and *S. brasiliensis* populations in Brazil. The distribution and diversity of haplotypes for *cal* plus *ef1* 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. schenckii* (seven) was calculated using the analysis of molecular variance (AMOVA) with Arlequin, version 3.11 (53). Φ_{ST} is analogous to the fixation index, F_{ST} , 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. schenckii* 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 *MAT1-1* locus in the *S. schenckii* genome (GenBank accession number [JX105435](#)) and a *MAT1-2* locus in the *S. brasiliensis* genome (GenBank accession number [JX101596](#)), 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. schenckii* 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. schenckii* scaffold (Fig. 1B).

Similar to other representatives in *Sordariomycetes*, the *S. schenckii* and *S. brasiliensis* *MAT* locus is flanked by *SLA2*, which encodes the cytoskeleton assembly control protein (ORFs SPSK05860 and SPBR04592), cytochrome *c* oxidase subunit *COX13* (ORFs SPBR05223 and SPSK06524), and DNA lyase *APN2* (ORFs SPSK05918 and SPBR04646) (55) (Fig. 2). The deduced amino acid sequences of *SLA2* and *APN2* shared, respectively, 89% and 62% identity with those of *Grossmannia clavigera*, a tree pathogen in the *Ophiostomatales* (39). Three open reading frames (ORFs) were predicted in the *MAT1-1* idiomorph of the *S. schenckii* 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 PPF domain and was 35% similar to *MAT1-1-2* of *O. montium* (GenBank accession number

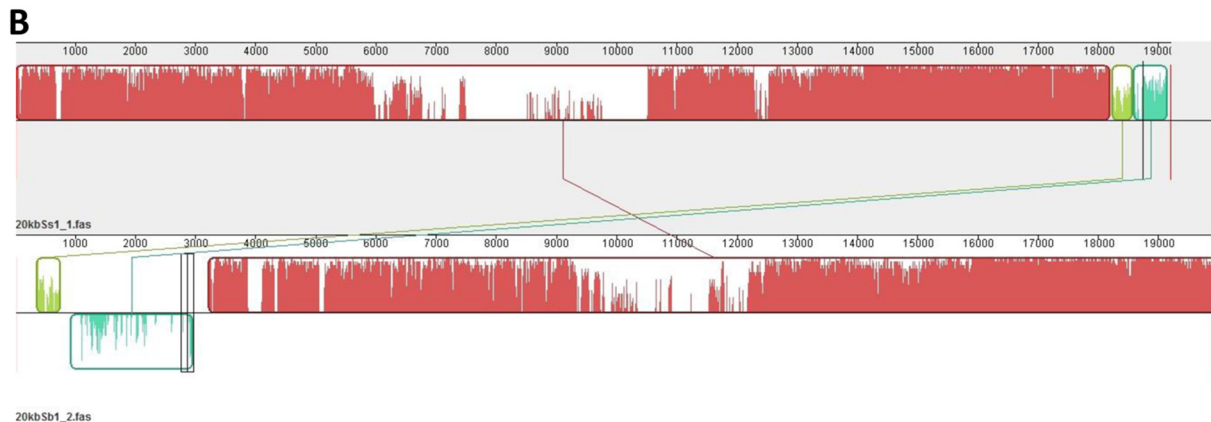
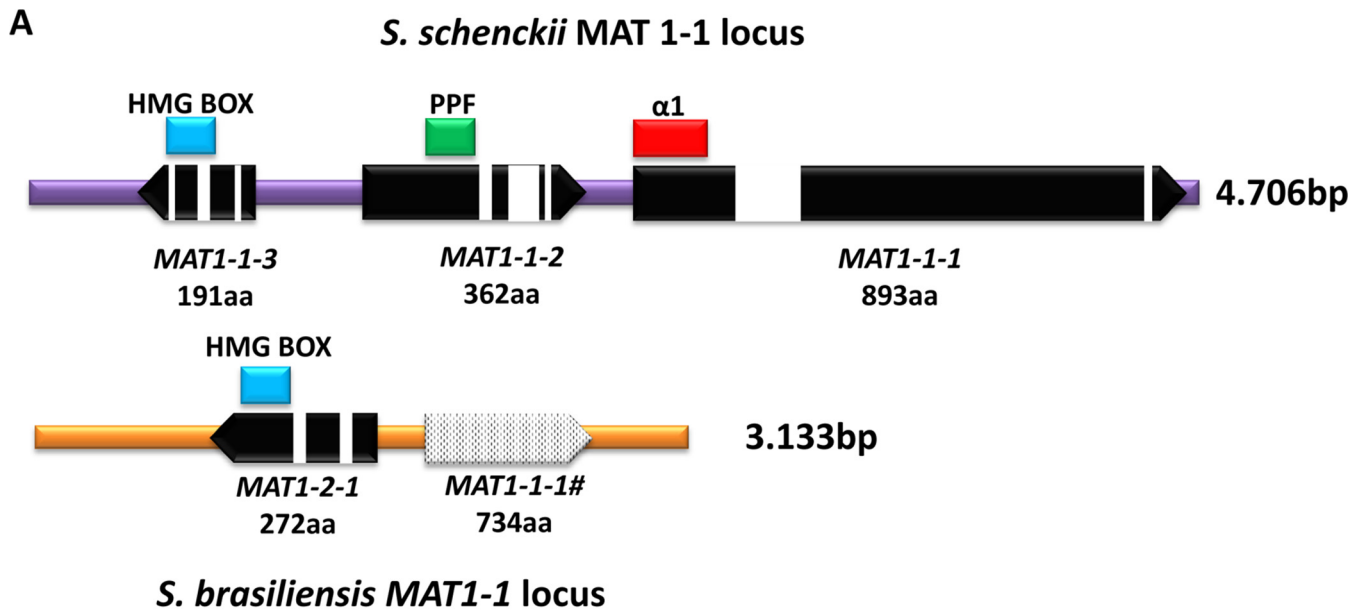


FIG 1 Genomic representation of mating type (*MAT*) locus of *S. schenckii* and *S. brasiliensis*. (A) Genomic analysis indicated that *S. schenckii* and *S. brasiliensis* harbor the *MAT1-1* (*MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3*, and *MAT1-2* (*MAT1-2-1*)) locus, as well its corresponding domains: $\alpha 1$, α -box domain; PPF, proline-proline-phenylalanine domain; HMG, high mobility group domain. A truncated *MAT1-1-1* was detected in the *S. brasiliensis* genome (*MAT1-1-1#*) showing the lack of the $\alpha 1$ domain. The protein product size and conserved domains as well intron distribution along the genes are shown. (B) Genomic alignment of a 20-kb *MAT*-containing scaffold between *S. schenckii* and *S. brasiliensis* showing the nonhomologous region corresponding to the *MAT* locus.

AGH03255.1). 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 α -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

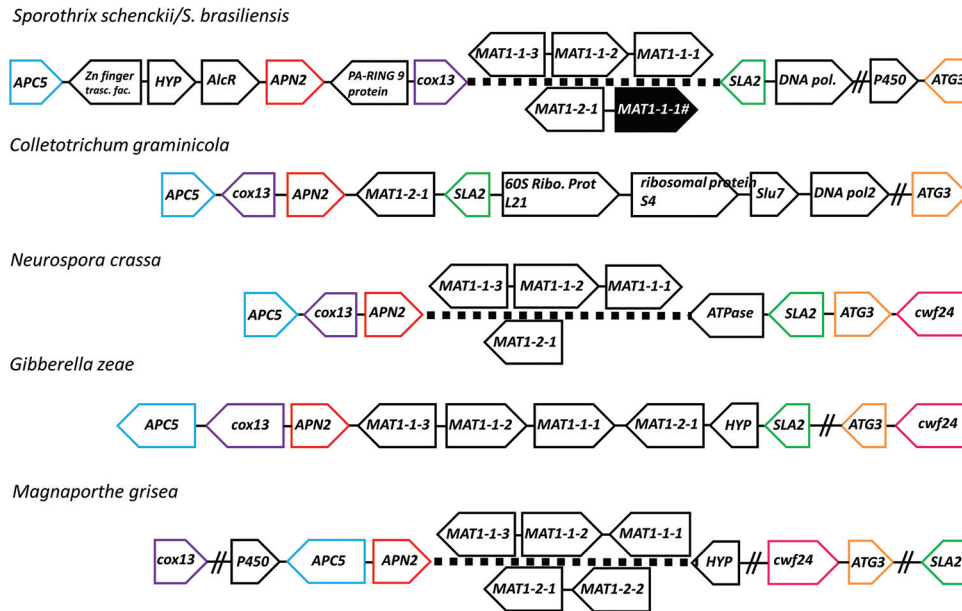


FIG 2 Syntenic organization of mating type (*MAT*) loci among *Sordariomycetes*. *MAT* genes are indicated by black arrows showing gene positions and orientation along the scaffolds. Overlapping genes presented in *MAT1-1* and *MAT1-2* loci are shown for heterothallic species, while both sets of *MAT* genes are shown in homothallic species. The surrounding genes of the *MAT* locus in *Sordariomycetes* are conserved. The *MAT1-1* or *MAT1-2* locus is flanked by *APC5*, *cox13*, and *APN2* on one side and by *SLA2* and *ATG3* on the other side. Ribo. Prot., ribosomal protein; pol, polymerase; trasc. fac., transcription factor. Double slashes indicate a span of over 50 kb.

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*, *MAT1-2-1*, 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 *MAT1-2-1* 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 rhamnogalacturonase B and muta-

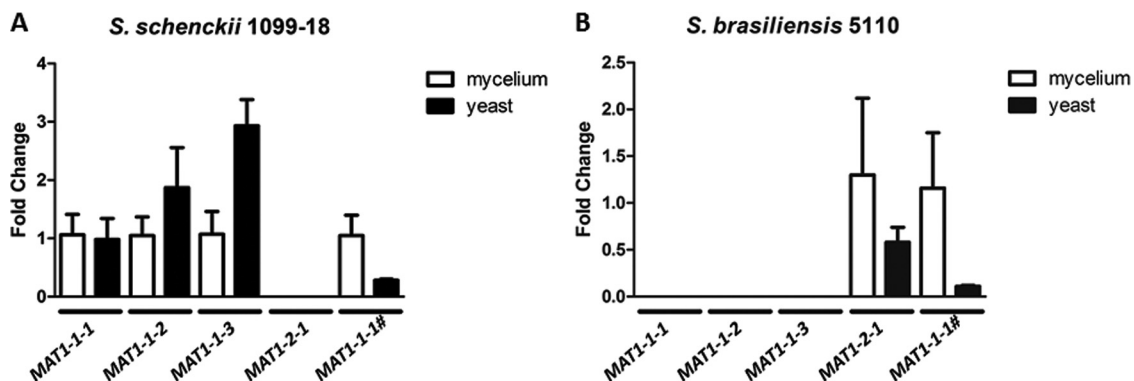


FIG 3 Quantitative expression of the *MAT1-1-1*, *MAT1-1-2*, *MAT1-1-3*, *MAT1-2-1*, and truncated *MAT1-1-1* (*MAT1-1-1#*) genes in *Sporothrix* species. Gene expression was measured between mycelium and yeast forms from *S. schenckii* (1099-18) and *S. brasiliensis* (5110).

TABLE 1 Mating type distribution of *Sporothrix* species

Species	No. of isolates by mating type		Chi-square value
	<i>MAT1-1</i>	<i>MAT1-2</i>	
<i>S. schenckii</i>	45	54	0.366
<i>S. brasiliensis</i>	37	53	0.092
<i>S. globosa</i>	2	2	1.000
Total	84	109	0.072

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

nase. 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 *MAT1-1-1* or *MAT1-2-1* 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 $\gg 0.05$) (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-*

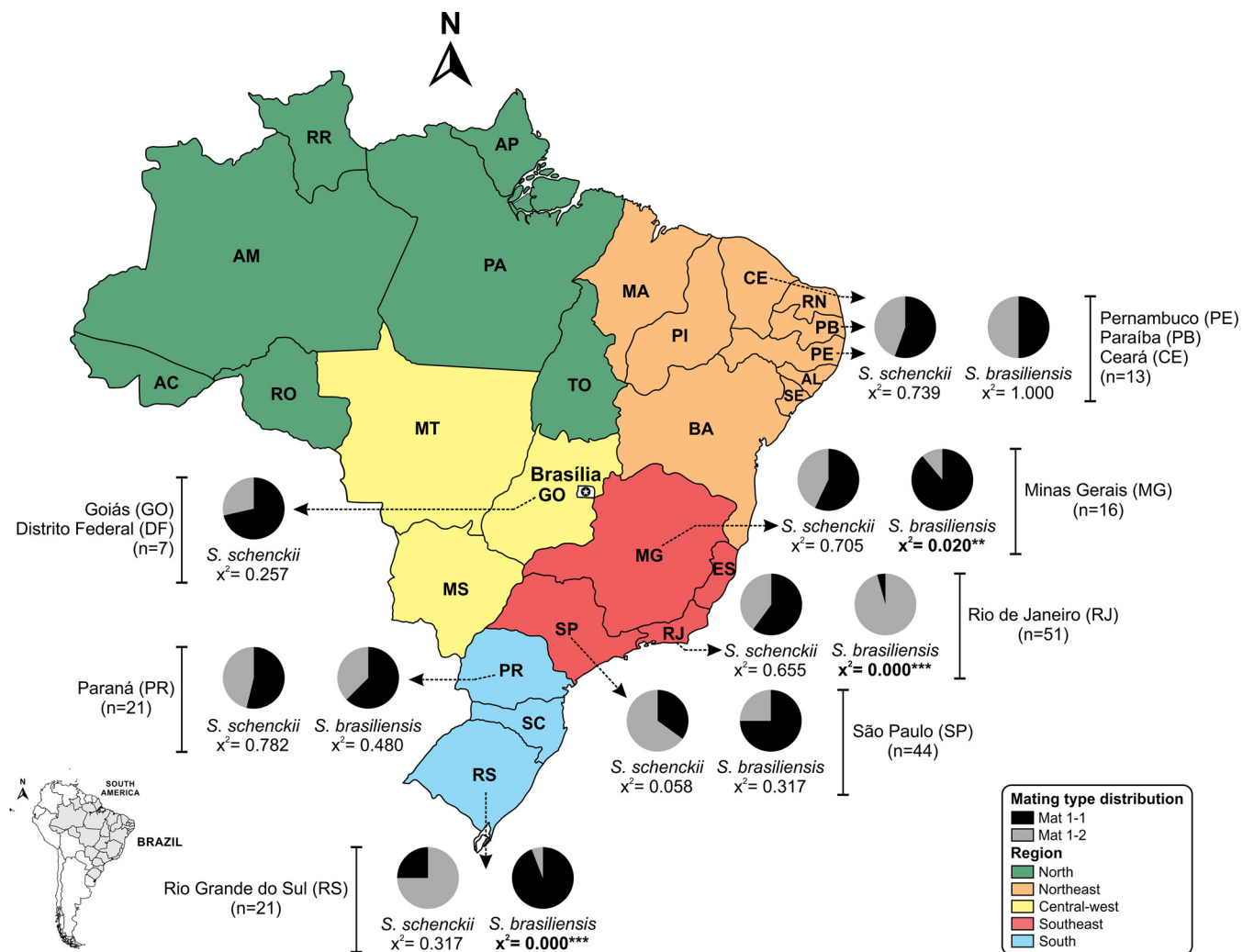


FIG 4 Geographic and mating type distribution of *S. schenckii* and *S. brasiliensis* isolates along the Brazilian territory discriminated by states. Mating type distributions (*MAT1-1* and *MAT1-2*) were tested for deviation from the expected ratios of 1:1 using a chi-square test, and P values were 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.

TABLE 2 Polymorphism analyses of *S. brasiliensis* and *S. schenckii* species

Parameter ^b	Value for the parameter at the indicated locus ^a			
	<i>S. schenckii</i>		<i>S. brasiliensis</i>	
	<i>cal</i>	<i>efl</i>	<i>cal</i>	<i>efl</i>
<i>S</i>	114	28	43	12
<i>H</i>	123	28	45	12
π	0.01177	0.00432	0.00243	0.00135
<i>h</i>	35	21	9	7
<i>h_d</i>	0.800	0.914	0.250	0.493
<i>k</i>	6.72300	3.01982	1.52196	0.94675
<i>M_{ed}</i>	0.012 (0.00.2)	0.008 (0.001)	0.002 (0.000)	0.001 (0.001)

^a Polymorphisms were evaluated using *cal* and *efl* loci and considering each species separately.

^b *S*, number of variable sites; *H*, total number of mutations; π , nucleotide diversity per site; *h*, number of haplotypes; *h_d*, haplotype diversity; *k*, average number of nucleotide differences; *M_{ed}*, mean evolutionary diversity for the entire population followed by its standard deviation.

ensis 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 (*H*), nucleotide diversity per site (π), number of haplotypes (*h*), haplotype diversity (*h_d*), average number of nucleotide differences (*k*), mean evolutionary diversity for the entire population (*M_{ed}*), and substitutions per site (*S_{ps}*) 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 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 adaptation 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. quercus* 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

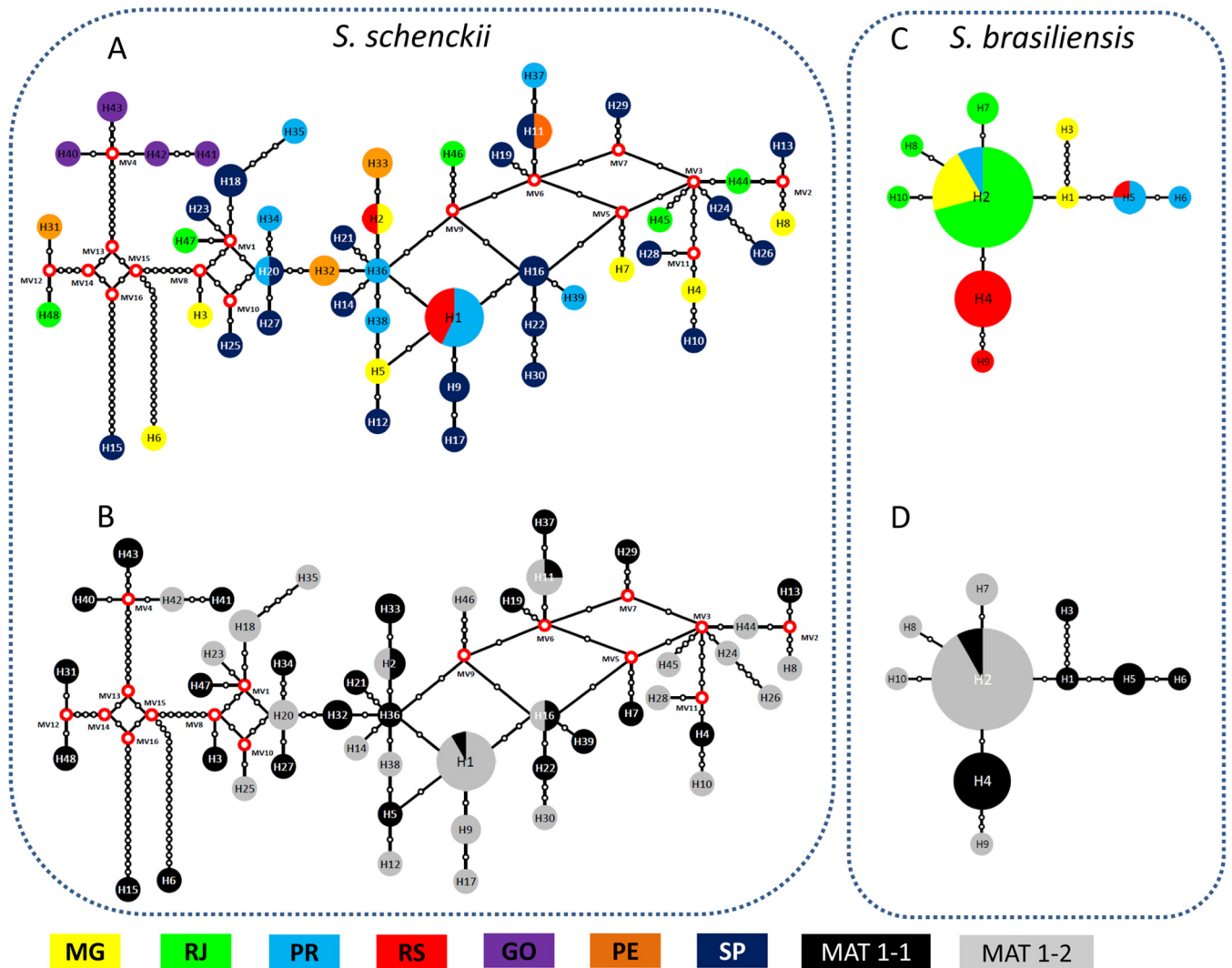


FIG 6 Median-joining haplotype network of *Sporothrix schenckii* (A and C) and *Sporothrix brasiliensis* (B and D) populations using concatenated nucleotide sequences of the calmodulin-encoding gene (*cal*) and elongation factor-1 alpha (*ef1*). Median vectors are displayed by red dots shown in the *S. schenckii* population (A and C). The mating type distribution was plotted in each haplotype of *S. schenckii* (C) and *S. brasiliensis* (D) populations. The circumference size is proportional to the haplotype frequency. MG, Minas Gerais; RJ, Rio de Janeiro; PR, Paraná; RS, Rio Grande do Sul; GO, Goiás; PE, Pernambuco; SP, São Paulo.

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 *S. brasiliensis* isolates (Fig. 5D to F). Recently, the pulsed-field gel electrophoresis (PFGE) profile of *S. brasiliensis* isolates showed less genome variability than *S. schenckii* isolates, indicating lower genomic rearrangements in *S. brasiliensis* than in *S. schenckii* (66).

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 *S. brasiliensis* may have more to do with *MAT* expansion. The fungus may have specific host interactions while *S. schenckii* 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 thermo-dimorphic 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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