Chromosomal Polymorphism in the *Sporothrix schenckii* Complex

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Abstract

Sporotrichosis is a polymorphic disease caused by a complex of thermomorphogenic fungi including *S. brasiliensis*, *S. schenckii sensu stricto* (*s. str.*), *S. globosa* and *S. luriei*. Humans and animals can acquire the disease through traumatic inoculation of propagules into the subcutaneous tissue. Despite the importance of sporotrichosis as a disease that can take epidemic proportions there are just a few studies dealing with genetic polymorphisms and genomic architecture of these pathogens. The main objective of this study was to investigate chromosomal polymorphisms and genomic organization among different isolates in the *S. schenckii* complex. We used pulsed field gel electrophoresis (PFGE) to separate chromosomal fragments of isolated DNA, followed by probe hybridization. Nine loci (β-tubulin, calmodulin, catalase, chitin synthase 1, Internal Transcribed Spacer, Pho85 cyclin-dependent kinase, protein kinase C Ss-2, G protein β subunit and topoisoamerase II) were mapped onto chromosomal bands of Brazilian isolates of *S. schenckii s. str.* and *S. brasiliensis*. Our results revealed the presence of intra and interspecies polymorphisms in chromosome number and size. 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. Our results bring new insights into the genetic diversity and genome organization among pathogenic species in the *Sporothrix schenckii* complex.

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Introduction

Sporotrichosis is a chronic, granulomatous, subcutaneous disease caused by several thermomorphogenic pathogenic species in the *Sporothrix schenckii* complex [1]. It is widely reported that the infection occurs through traumatic inoculation of fungal propagules into the skin by contaminated material, such as soil, thorns or splinters. Sporotrichosis mainly affects humans and animals [2–4]. The zoonotic potential, as exemplified by animal scratches and bites, particularly from cats, are the most common modes of transmission to humans in hyperendemic areas in Brazil [3,6]. In some cases, human infections are associated with transmission from wild animals; for example, injuries caused by armadillos when hunting the animal [7]. Sporotrichosis has a worldwide distribution with a high incidence in temperate and tropical regions including Latin America (Brazil, Mexico, Colombia, Costa Rica, Guatemala, and Uruguay), South Africa, India, and Japan [8].

Currently, medically relevant *Sporothrix* spp. in the *S. schenckii* complex are *S. brasiliensis* (Clade I), *S. schenckii sensu stricto* (*s. str.*) (Clade II), *S. globosa* (Clade III), and *S. luriei* (Clade VI) [1]; while, *S. mexicana* (Clade IV) and *S. pallida* (Clade V) are remotely related in phylogenetic analysis [1,3,6] and, therefore, are considered apart from the clinical group. These species were identified based on multi-locus sequencing, morphology, and physiological characteristics [9,10]. *Sporothrix schenckii s. str.* is found predominantly on the American, Asian, and African continents; *S. globosa* is a widespread species, found with high frequency in Europe and Asia; and *S. brasiliensis* has been isolated exclusively in Brazil [1,9]. *Sporothrix luriei* is a rare pathogen. Four human cases have been reported, but the etiological agent was only isolated in the first reported case [11]. In Brazil, over the last decades, the incidence of sporotrichosis has increased exponentially to epidemic proportions. The epidemic occurred mainly in Rio de Janeiro, and was caused by *S. brasiliensis* [6,9].

Recent studies have shown important differences in virulence and drug resistance profiles among different species in the *S. schenckii* complex. *Sporothrix brasiliensis* is the most virulent species. In contrast, *S. globosa* and *S. mexicana* showed little or no virulence in a murine model [12–14]. When compared for drug resistance,
S. brasiliensis was less resistant than other species, and S. mexicana was the most resistant [15]. Despite these findings, little is known about the genetic mechanisms of virulence and drug resistance in the S. schenckii complex.

Fungi present complex genomes and fungal chromosomes are small and difficult to visualize with traditional karyotyping techniques [16]. The development of pulsed field gel electrophoresis (PFGE) by Schwartz and Cantor [17] and improvements of this technique over the years have promoted a novel method for studying fungal chromosomes. With this method, the molecular karyotypes of eight S. schenckii sensu lato (s.l.) isolates were defined in Japan. Those isolates appeared to include 6 to 8 chromosomal bands, and each band contained one or more chromosomes of the same size. The genome size was estimated to be approximately 28 Mb [18]. In that study, the authors applied four distinct conditions to separate and estimate the chromosome numbers and sizes for S. schenckii s.l. isolates. The four different conditions used made it difficult to compare similar chromosomes, and that approach may not have provided accurate estimates of the genome size. Furthermore, that study was performed before the new Sporobolomyces species had been defined; hence, it is not clear whether the authors studied S. schenckii s. str. or isolates of S. globosa, which are also found in the Asian territory.

The genomic organization and chromosome number in different species in the S. schenckii complex remain unknown. The aim of the present study was to investigate chromosomal polymorphisms among Sporobolomyces spp. isolates from diverse geographic origins with the PFGE technique. Additionally, we used hybridization to map the location of nine genetic markers onto the chromosomal bands of Brazilian isolates of S. schenckii s. str. and S. brasiliensis. Our results showed the existence of polymorphisms in the number and size of chromosomes among different Sporobolomyces isolates. This information will be useful for genetic mapping and for future studies on the chromosomal organization and epidemiology of the S. schenckii complex.

Materials and Methods

Fungal Strains and Growth Conditions

Fungal isolates (n = 23) were grown on Sabouraud agar slants at room temperature for 7 days. Then, the total growth from each slant was transferred to a 500 mL Erlenmeyer flask containing 50 mL of Brain Heart Infusion (BHI) broth (Acumedia, USA), supplemented with dextrose (18 g/L), pH 8.0, and incubated at 37°C for 10 days, under gentle shaking. This procedure induced yeast formation. The codes and information for all isolates are summarized in Table 1.

Isolation of Genomic DNA and Preparation of Intact Chromosomal DNA from Sporobolomyces spp. Cells

Genomic DNA was extracted according to the protocol of de Bievre et al. [19], with some modifications. Briefly, yeast cells were washed 3 times with wash buffer (50 mM EDTA, pH 8.0); then, cells were resuspended in protoplast buffer, which contained 5 mM EDTA, 100 mM Tris-HCl pH 7.5, 0.01% (v/v) β-mercaptoethanol, and 0.1 mg/mL zymolyase-20T (MP Biomedicals, USA). Cells were incubated at 37°C for 1 h. Next, lysis buffer was added, which contained 10 mM Tris-HCl, 1 mM EDTA, 1 mg/mL proteinase K, 5% (w/v), and sarcosyl. Cells were incubated for 1 h at 56°C. After centrifugation, the pellet was washed with 70% ethanol dried, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), supplemented with 1 μg/mL RNase. The solution was incubated for 1 h at 37°C. The remaining DNA in solution was washed with phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged. The water phase was collected, isopropl alcohol was added, and the suspension was incubated for 16 h at −20°C. Finally, the samples were centrifuged, and the pellet was washed with 70% ethanol. The pellet was allowed to dry at 37°C, then resuspended in sterile water, and stored at −20°C until use.

We embedded cells within agarose plugs that could be inserted into a PFGE system for chromosome isolation. PFGE plugs were prepared according to the protocol of Herschleb et al. [20], with minor modifications. Briefly, yeast cells were harvested from BHI broth and washed 3 times with wash buffer (EDTA 50 mM, pH 8.0); 2 × 10⁵ yeast cells were used to prepare each agarose plug. The cells were mixed with Zymolyase solution (1 mg/mL Zymolyase-20T in glycerol-phosphate buffer, pH 7.0) and 1% Low Melting Point Agarose (FMC BioProducts, USA) to achieve a final concentration of 1 × 10⁶ cells/mL. Then, the cell suspension was pipetted into a casting mold, and the agar was allowed to solidify at 4°C. The plugs were removed from the molds, and a spheroplast solution (0.5 M EDTA, 7.5% β-mercaptoethanol, pH 8.0) was added. The PFGE plugs were incubated overnight at 37°C. The spheroplast solution was replaced with NDSK buffer, which contained 0.5 M EDTA, 1% (w/v) X-lauysarcosine (Sigma, USA), and 1 mg/mL Proteinase K (Sigma, USA), pH 9.5. Then, the PFGE plugs were washed 3 times with wash buffer, and incubated overnight at 50°C. After washing 3 times with wash buffer, the NDSK buffer was replaced with stock solution (0.5 M EDTA, pH 9.5) and stored at 4°C until use.

Pulsed Field Gel Electrophoresis

The PFGE separation was performed according to Feitosa et al. [21], with minor modifications. Briefly, DNA plugs were loaded onto the PFGE gel (0.8%), and chromosomal separation was carried out in the Gene Navigator System (Amersham Pharmacia Biotech, USA) at a constant temperature of 10°C. The best chromosomal band resolution was achieved in 1 x TAE buffer with homogeneous pulses and interpolation for 168 h at 42 V (phase 1: pulse time 900 s/24 h; phase 2: 1800 s/24 h; phase 3: 2700 s/48 h; phase 4: 3600 s/48 h; phase 5: 5400 s/24 h). Schizosaccharomyces pombe chromosomes (Bio-Rad, USA) were run in parallel as standard size markers. After electrophoresis, the gel was stained with 0.5 μL/mL ethidium bromide (0.5 μL/mL; EtBr) for 15 min, and washed with distilled water for 15 min. The digital images of the gel were acquired with a L-Pix Touch documentation system (Locusc Biotecnologia, Brazil).

Densitometric Analysis

Densitometric analysis was performed with LabImage 1D, ver. 3.3.4 software (Kapelan Bio-Imaging, Germany), and chromosomal band sizes were estimated. Each chromosomal band size was calculated by comparing its migration with that of S. pombe chromosomes, which were used as standard size markers.

PCR Amplification and DNA Probes

PCR was used to amplify gene fragments from β-tubulin (β-tub) [10], calmodulin (Calm) [22], catalase (Cata) [23], chitin synthase I (CHSI) [24], Internal Transcribed Spacer (ITS) [25], Pho55 cyclin-dependent kinase (Pho55) [26], protein kinase C δs-2 (PKCδ) [27], G protein δ subunit (GProt) [28], and topoisomerase II (Topo) [29] with primers designed to target specific gene
sequences (Table S1). The DNA sample from *S. schenckii* *s. str.* (Ss126) (100 ng/μL) was also amplified in PCR reactions with the PCR Master Mix (Promega, USA), according to the manufacturer’s protocols. Each amplicon was cloned into the pGEM-T Easy Vector System (Promega, USA), following the manufacturer’s instructions. These plasmids were transfected into Subcloning

### Table 1. Strains, species, origin, and GenBank accession numbers (CAL and ITS) for the Sporothrix spp. isolates used in this study.

| Isolate | Other Code | Species  | Source | Country | CAL    | ITS    | Reference |
|---------|------------|----------|--------|---------|--------|--------|-----------|
| IPEC 16919 | FMR 8314, Ss177 | *S. brasiliensis* | Human | Brazil | AM116899 | KF574441 | 9, 10, This study |
| CBS 120339 | IPEC 16490, Ss178 | *S. brasiliensis* | Human | Brazil | AM116898 | KF574440 | 9, 10, This study |
| ATCC MYA-4823 | CBS 132021, Ss322 | *S. brasiliensis* | Feline | Brazil | KF574459 | JQ070114 | This study |
| Ss99 | – | *S. brasiliensis* | Human | Brazil | KF574460 | KF574442 | This study |
| Ss104 | – | *S. brasiliensis* | Human | Brazil | KF574461 | KF574443 | This study |
| Ss52 | – | *S. brasiliensis* | Human | Brazil | KC693845 | KF574444 | 6, This study |
| Ss54 | CBS 132990 | *S. brasiliensis* | Feline | Brazil | JQ041903 | JN885580 | 3, 14 |
| CBS 130106 | IPEC 15572 | *S. brasiliensis* | Human | Brazil | AM116886 | FNS49903 | 1, 9, 10, 32 |
| CBS 130108 | IPEC 17943 | *S. brasiliensis* | Human | Brazil | AM116878 | FNS49902 | 1, 9, 10, 32 |
| Ss265 | CBS 133020 | *S. brasiliensis* | Human | Brazil | JN204360 | JN885580 | 3, 6 |
| CBS 130109 | IPEC 22582 | *S. brasiliensis* | Human | Brazil | AM116874 | KF574445 | 3, 6, This study |
| CBS 130107 | IPEC 17521 | *S. brasiliensis* | Human | Brazil | AM116874 | KF574445 | 3, 6, This study |
| CBS 359.36 | Ss185 | *S. schenckii* | Human | USA | AM117437 | FJ545232 | 9, 10, 32 |
| Ss03 | CBS 132963 | *S. schenckii* | Human | Brazil | JX071117 | KF574446 | 3, This study |
| Ss39 | – | *S. schenckii* | Human | Brazil | JQ041989 | JN885576 | 6, 14 |
| Ss51 | – | *S. schenckii* | Human | Brazil | JQ041902 | JN885579 | 14 |
| Ss61 | – | *S. schenckii* | Environmental | Brazil | KF561244 | KF574447 | This study |
| Ss126 | – | *S. schenckii* | Human | Brazil | JQ041904 | JN885581 | 14 |
| Ss137 | – | *S. schenckii* | Human | Brazil | KF574462 | KF574448 | This study |
| Ss140 | – | *S. schenckii* | Human | Brazil | KF574463 | KF574449 | This study |
| Ss141 | CBS132975 | *S. schenckii* | Human | Brazil | JQ041905 | JN885582 | 14 |
| Ss159 | CBS 132976 | *S. schenckii* | Human | Brazil | KF574464 | KF574450 | This study |
| Ss160 | – | *S. schenckii* | Human | Mexico | KF574465 | KF574451 | This study |
| Ss161 | – | *S. schenckii* | Human | Mexico | KF574466 | KF574452 | This study |
| Ss162 | CBS 132977 | *S. schenckii* | Environmental | Mexico | KF574467 | KF574453 | This study |
| Ss163 | – | *S. schenckii* | Human | Peru | KF574468 | KF574454 | This study |
| Ss164 | – | *S. schenckii* | Human | Peru | KF574469 | KF574455 | This study |
| ATCC MYA-4821 | CBS 132984, Ss323 | *S. schenckii* | Human | USA | KF574470 | JQ070112 | This study |
| Ss40 | – | *S. schenckii* | Human | Brazil | JQ041900 | JN885577 | 14 |
| Ss143 | – | *S. schenckii* | Human | Brazil | JQ041906 | JN885583 | 6, 14 |
| CBS 120340 | FMR 8600 | *S. globosa* | Human | Spain | AM116908 | FNS49905 | 9, 10, 32 |
| Ss06 | CBS 132922 | *S. globosa* | Human | Brazil | JF811336 | JN885574 | 3, 6 |
| Ss41 | CBS 132923 | *S. globosa* | Human | Brazil | JF811337 | KF574456 | 3, 6, This study |
| CBS 130116 | FMR 8598 | *S. globosa* | Human | Spain | AM116903 | KC113226 | 9, 10, 1 |
| CBS 130104 | FMR 8595 | *S. globosa* | Human | Spain | AM116905 | KC113225 | 9, 10, 1 |
| CBS 130105 | FMR 8597 | *S. globosa* | Human | Spain | AM116907 | FNS49904 | 9, 10, 32 |
| CBS130117 | FMR 9022 | *S. globosa* | Human | Japan | AM398995 | KC113229 | 9, 1 |
| CBS 130115 | FMR 8596 | *S. globosa* | Human | Spain | AM116902 | KC113228 | 9, 10, 1 |
| CBS 937.72 | ATCC 18616, Ss187 | *S. luriei* | Human | South Africa | AM743702 | AB128012 | 11 |
| CBS 120341 | FMR 9108, Ss182 | *S. mexicana* | Environmental | Mexico | AM398933 | FNS49906 | 9, 32 |
| Ss132 | CBS 132927 | *S. mexicana* | Human | Brazil | JF811340 | KF574457 | 3, 6 |
| Ss327 | PG3 | *S. pallida* | Feline | Brazil | KF574471 | KF574458 | This study |
| CBS 124561 | FMR 9338 | *S. brunneoviolacea* | Environmental | Spain | KF574472 | FNS46959 | 32 |

IPEC, Instituto de Pesquisa Clinica Evandro Chagas, Fiocruz, Brazil; FMR, Facultat de Medicina i Ciències de la Salut, Reus, Spain; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; ATCC, American Type Culture Collection, Manassas, VA, USA; T, type strain. All “Ss” strains belong to the culture collection of the Federal University of São Paulo (UNIFESP).

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Chromosomal Polymorphism and *S. schenckii* Complex
Efficiency DH5-α Competent Cells (Invitrogen, USA) according to the manufacturer’s protocol. Plasmids were purified with the NucleoSpin Plasmid Kit (Macherey-Nagel, Germany) and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI 3730 DNA Analyzer (Applied Biosystems, USA).

Radiolabeling Probes and Southern Blot Hybridization

Specific probe sequences were prepared by excising DNA fragments from the plasmids with the EcoRI restriction enzyme (Fermentas, USA), according to the manufacturer’s instructions. The probes were purified with the NucleoSpin Extract II kit (Macherey-Nagel, Germany). In addition, the entire 7.0 Mb chromosomal band of isolate Ss126 was excised from an agarose gel, and the agarose was digested with β-agarase (Lonza, USA), according to the manufacturer’s protocol. Also, genomic DNA was digested with the HaeIII restriction enzyme (Fermentas, USA), according to the manufacturer’s instructions, then the fragments were purified to use as a probe. All probes were radiolabeled with the Random Primers Labeling System kit (Invitrogen, USA) and dCTP (α-32P) (Perkin Elmer, USA).

After PFGE, chromosomal bands were transferred to a Hybond-N nylon membrane (Amersham Bioscience, United Kingdom) by capillary action at room temperature. DNA was fixed to the membrane in a GS Gene Linker UV™ Chamber (Bio-Rad, USA) under UV irradiation (150 mJ). The fixed membranes were stored at −4°C until use.

For hybridization, the membranes were incubated with prehybridization buffer, which contained 0.25 M Na2PO4, 2 mL phosphoric acid (85%), 7% SDS, 1% BSA, and 1 mM EDTA, for 30 min at 65°C. The radiolabeled probes were added, and hybridization was carried out at 65°C, for 16 h. The membranes were washed once in 20 ml of Solution I (0.25 M Na2PO4, 2 mL phosphoric acid, 1% SDS, and 1 mM EDTA) at 65°C for 30 min, and then once in 20 ml of solution II (0.125 M Na2PO4, 1 mL phosphoric acid [85%], 1% SDS, and 1 mM EDTA) for 30 min.

Next, the membranes were exposed to x-ray film (GE Healthcare, United Kingdom) for 2 to 4 days at −80°C, and finally, the film was developed.

Phylogenetic Analysis

The calmodulin gene [9] and ITS region fragments [1] were amplified directly from genomic DNA with primer sets CL1 and CL2A (for calmodulin) and ITS1 and ITS4 (for ITS). Amplified products were gel-purified with the Wizard SV Gel and PCR Clean-Up System (Promega Corp., USA), according to the manufacturer’s instructions. DNA samples were sequenced as described above. Fragments were sequenced on both strands to increase the quality of sequence data (Pherd≥30). Alignment of sequences was performed with the ClustalW algorithm [30], implemented in BioEdit software [31]. Retrieved alignments were manually corrected to avoid mispaired bases. Nucleotide sequences were exported into a FASTA file to use in a BLAST search (www.ncbi.nlm.nih.gov/BLAST).

For reference sequences, this study included calmodulin sequences (n = 43) from other isolates of S. schenckii s. str. (n = 8), S. brasiliensis (n = 9), S. globosa (n = 8), and S. mexicana (n = 2), and ITS sequences (n = 43) from isolates of S. schenckii s. str. (n = 8), S. brasiliensis (n = 6), S. globosa (n = 7), S. mexicana (n = 1), and S. brunnewoldiae (n = 1). These sequences were previously deposited in GenBank (www.ncbi.nlm.nih.gov/genbank) and described by Marimon et al. [9,10], Rodrigues et al. [3,6], Fernandes et al. [14], Madrid et al. [32], Zhou et al. [1], Oliveira et al. [33], and Silva-Vergara et al. [34] (Table 1). The calmodulin and ITS sequences were concatenated with FaBox software [35], available at http://www.birc.au.dk/fabox. Evolutionary analysis was performed in MEGA5 [36] with Neighbor-joining and Maximum Likelihood methods. To estimate confidence values for individual clades, the Tamura 3-parameter nucleotide substitution model was used [37], with a gamma distribution (shape parameter = 1) rate variation among sites, and 1,000 bootstrap replicates [38].

Ethics Statement

The procedures performed in this study were approved by Research Ethics Committee of UNIFESP [CEP N°. 0111/11].

Results

Phylogenetic Analysis

The Calm and ITS regions were amplified with PCR, which yielded fragments of approximately 800 bp and 620 bp, respectively. These sequences were aligned and concatenated for phylogenetic analysis. The concatenated sequences were 1,389 bp long and included 872 invariant characters (63%), 224 variable parsimony-informative characters (16%), and 183 singleton characters. Positions that contained gaps or missing data were eliminated. The 43 concatenated sequences were clustered into 7 main groups. Clades I–III and VI (S. brasiliensis, S. schenckii s. str., S. globosa and S. luriei, respectively) are related to human and animal infections, while S. pallida (Clade V), S. mexicana (Clade IV) and S. brunnewoldiae are well described as soil and decaying wood inhabiting (Fig. 1).

In general, S. schenckii (Clade II) isolates were highly diverse; most isolates from Brazil preferentially clustered into the subclade opposite to the type strain (CBS 359.36). However, the isolates from Mexico and Peru were distributed through the clade II. On the other hand, clade I (S. brasiliensis) and clade III (S. globosa) appeared to be more homogeneous, with low genetic diversity.

Molecular Karyotype and Chromosomal Polymorphisms in the S. schenckii Complex

The chromosomal bands of Spondylospora spp. isolates were separated by PFGE and stained with EtBr. A visible chromosomal band on the PFGE may contain one or more co-migrating chromosomes which are not necessarily homologous.

In the densitometric analysis of chromosomal bands separated with PFGE, we determined the numbers and sizes of chromosomes among 16 isolates of the S. schenckii complex. The different S. schenckii s. str. isolates displayed remarkable differences in chromosome profiles. The isolates had 4 to 7 chromosomal bands, ranging from 2.0 to 7.0 Mb (Fig. 2A, C; Figs. S1 and S2). In contrast, S. brasiliensis isolates showed fewer polymorphisms; in which the karyotype was composed of 5 to 7 chromosomal bands, ranging from 2.9 to 7.0 Mb (Fig. 2B, D; Fig. S3). Interestingly, several isolates of S. schenckii s. str. and S. brasiliensis had similar electrophoretic karyotypic profiles (Fig. 2A and Fig. S1A-C; Fig. 2D and Fig. S3A, C, respectively). The isolates of S. mexicana and S. luriei had karyotype profiles composed of 4 (ranging from 4.0 to 7.0 Mb) and 6 (ranging from 1.2 to 7.0 Mb) chromosomal bands, respectively (Fig. 2E–F).

To facilitate visualization and comparisons among the karyotype profiles of all isolates from the S. schenckii complex, we created a schematic of chromosomal bands (Fig. 3). Most chromosomal bands of S. schenckii s. str. and S. brasiliensis isolates ranged in size from 7.0 to 3.8 Mb and from 3.3 to 2.5 Mb. Several isolates also had bands ranging from 5.7 to 3.8 Mb. Spondylospora mexicana had no chromosomal bands smaller than 4.0 Mb, but the S. luriei isolate had a small chromosomal band of 1.2 Mb. The electrophoretic
karyotypes were not correlated with the source of isolation (i.e., human or environment) or with the clinical characteristics of the disease.

Mapping *S. schenckii* s. str. Genes in Chromosomal Bands of Different Isolates

Due to the high prevalence of *S. brasiliensis* and *S. schenckii* s. str. in Brazil [3,6,9], we evaluated the differences in genome structures among eight *S. schenckii* s. str. isolates and two *S. brasiliensis* isolates collected in distinct regions of Brazil. The hybridization profiles generated with nine genetic markers were very similar among the *S. schenckii* s. str. and *S. brasiliensis* isolates. Some probes, like Calm, CHS1, ITS, GProt, PKC, Pho85, and Topo, hybridized preferentially with the large chromosomal bands (Fig. 4A–G). On the other hand, other probes, like β-tub and Cata, hybridized preferentially with small chromosomal bands (Fig. 4H–I).

The probe hybridization patterns on chromosomal bands are shown schematically in Figure 5. The 6.5 Mb chromosomal band hybridized with 3 to 6 probes. Four isolates of *S. schenckii* showed a similar pattern of hybridization (Ss51, Ss137, Ss126 and Ss03) on the 6.5 Mb chromosomal band; in figure 5 the colors indicate the six different probes that hybridized with this band: Calm, CHS1, ITS, PCK, GProt, and Topo. Interestingly, probe Pho85 hybridized with the 5.8 Mb chromosomal band in all isolates. The ITS probe hybridized with small chromosomal bands in *S.
brasiliensis isolates, but with large chromosomal bands in S. schenckii s. str. In addition to this general observation, it is interesting to note that some chromosomal polymorphisms were detected, mainly in the S. schenckii s. str. isolates. We identified several possible events, including translocation and duplication.

Mapping the chromosomal bands with the catalase probe yielded weak hybridization signals in isolates Ss140 and Ss39. Those signals could be clarified when the Hybond nylon membrane was subjected to longer exposure times. The same occurred with PKC in isolates Ss137, Ss126, and Ss03 (Fig. 4E, I). We also observed that Calm, GProt, and ITS probes hybridized with two chromosomal bands in the Ss39 isolate; Calm and GProt hybridized with the 6.7 Mb and 4.7 Mb bands, and ITS hybridized with the 6.7 Mb and 5.8 Mb bands. This suggested the possibility that a gene duplication event may have occurred in this isolate (Fig. 5). In addition, a comparison of the hybridization profiles showed that Calm and GProt were present in all isolates. It is tempting to suggest that those two genes may constitute a linkage

Figure 2. Densitometric analysis of S. schenckii, S. brasiliensis, S. mexicana, and S. luriei isolates. Each panel shows (left) the ethidium bromide-stained gel after pulsed field gel electrophoresis of chromosomes from the fungus strain indicated, and (right) a graph of the densitometric analysis. The size of each chromosomal band (Mb) is indicated on the left and above the corresponding peaks on the graph. Open arrows indicate where samples were loaded.

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group; therefore, they may be two parts of the same DNA molecule. Another possible gene duplication event occurred in the Ss03 isolate; this involved the β-tubulin gene which hybridized to two chromosomal bands of 3.5 Mb and 2.7 Mb. Whether only the gene locus or the entire chromosome was duplicated remains to be demonstrated.

We also identified a possible translocation event of PKC gene. In Ss141 isolate, the PKC probe strongly hybridized to the 2.7 Mb chromosomal band while in others isolates it hybridized on larger size bands (6.5 Mb, 6.3 Mb and 5.4 Mb). Other genes (CHS1, Topo, GProt and Calm) that were mapped with PKC on larger size bands did not move to 2.7 Mb band (Fig. 4), supporting the hypothesis above. Despite the previously reported [39] diploid nature of S. schenckii s.l., our results suggested that this organism may be aneuploid for several chromosomes.

To establish a correlation among the 7.0 Mb chromosomal bands observed in all isolates, this band from isolate Ss126 was excised from the gel, radiolabeled with 32P, and hybridized to the chromoblots. The 7.0 Mb probe hybridized preferentially with the large chromosomal bands in S. schenckii isolates. This hybridization pattern suggested the presence of repetitive sequences in S. schenckii s.l., our results suggested that this organism may be aneuploid for several chromosomes.

Estimation of Genome Size

The minimum size of a genome was estimated as the sum of the individual chromosomal bands. The minimum genome size of S. schenckii s. str. isolates varied from 25.1 to 37.5 Mb, and that of S. brasiliensis isolates varied from 25.7 to 34.7 Mb (Fig. 3). The average genome sizes of S. schenckii s. str. and S. brasiliensis were 29.3 Mb and 27.4 Mb, respectively; the genome sizes of S. mexicana and S. luriei isolates were 22.1 Mb and 26.8 Mb, respectively (Fig. 6A).

Based on genomic data from the genome sequencing project of S. schenckii s. str. (ATCC MYA-4821) and S. brasiliensis (ATCC MYA-4823) isolates, the genome sizes of ATCC MYA-4821 and ATCC MYA-4823 were estimated to be 32.5 Mb and 33.4 Mb, respectively (Marcus de Melo Teixeira and Maria Sueli Soares Felipe, personal communication). In the present study, the minimum genome sizes evaluated by PFGE of these isolates were estimated to be 25.3 Mb (ATCC MYA-4821) and 25.7 Mb.
Thus, the genome sizes based on genome sequencing were larger than those based on the sum of chromosomal bands (Fig. 6B).

Densitometric analyses showed that both the ATCC MYA-4821 and ATCC MYA-4823 isolates had very high peak pixel intensities for the 7.0 Mb chromosomal band. The 7.0 Mb band was also strongly stained with EtBr (Figure S2A and Figure S3A).

Figure 4. Specific gene probes hybridized to chromosomal bands of Brazilian isolates of *S. schenckii* and *S. brasiliensis*. Southern blots show radiolabeled probes hybridized to fungi chromosomes. Probes that specifically targeted A: calmodulin (Calm), B: chitin synthase 1 (CHS1), C: Internal Transcribed Spacer (ITS), D: G protein a subunit (GProt), E: protein kinase C Ss-2 (PKC), F: Pho85 cyclin-dependent kinase (Pho85), and G: topoisomerase II (Topo) hybridized preferentially to large bands in most isolates. Probes for H: l-tubulin (l-tub) and I: catalase (Cata) hybridized preferentially to small bands in most isolates. J: The 7.0 Mb chromosomal band from isolate Ss126 was used as a probe. The hybridization pattern shows evidence for repetitive sequences. The sizes of major chromosomal bands (Mb) are indicated on the left. The open arrows indicate the wells where samples were loaded. Areas enclosed in a dashed box (E and I) are lanes that were exposed for longer times to better visualize the hybridization signals.

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ATCC MYA-4821 and ATCC MYA-4823, respectively). In fact, the pixel intensities of the 7.0 Mb chromosomal band (113 for ATCC MYA-4821 \textit{S. schenckii} and 80 for ATCC MYA-4823 \textit{S. brasiliensis}), were close to twice that of the 6.5 Mb chromosomal band (66 and 54 for ATCC MYA-4821 and ATCC MYA-4823, respectively; data not shown). This observation suggested the possibility that an additional chromosome may be present in the 7.0 Mb chromosomal band. This might explain the difference in genome size estimations discussed above. For example, when a second 7.0 Mb chromosome band was added to the sum of all chromosomal bands, the minimum genome sizes were 32.3 Mb and 32.7 Mb for ATCC MYA-4821 and ATCC MYA-4823, respectively. Therefore, our estimations of genome sizes would be very close to those reported from genome sequencing. Taken together, our results strongly suggested that the 7.0 Mb chromosomal band might contain two or more chromosomes for the ATCC MYA-4823 and ATCC MYA-4821 isolates.

Another indication of chromosomal band co-migration arose from the \textit{S. schenckii} \textit{s. str.} gene mapping. The hybridization analysis suggested that the 6.5 Mb chromosomal band might harbor two co-migrating chromosomes. In most \textit{S. schenckii} isolates, the probes Calm, ITS, CHS1, PKC, GProt, and Topo hybridized with the 6.5 Mb chromosomal band; however, in isolates Ss61 and Ss39, those probes hybridized with two chromosomal bands that ranged from 6.3 to 6.7 Mb (Figs. 4 and 5). The presence of a second 6.5 Mb chromosomal band would increase the average minimum genome size of the \textit{S. schenckii} isolates to 32.0 Mb (Ss51, Ss137, Ss126, and Ss05) and 37.2 Mb (Ss141). Those values were very close to those reported from genome sequencing, except for the Ss141 isolate, where we estimated a size that was approximately 5.0 Mb higher than the size they estimated. This difference could be explained by karyotype polymorphisms that were previously described for this species.

**Discussion**

Based on the phylogenetic analysis with sequences from Calm and ITS, we were able to differentiate among the species in the \textit{S. schenckii} complex. In this study, we found tree topology that was similar to that reported in other studies that used only calmodulin sequences [3,6,9,11,32]. The electrophoretic karyotype profiles of \textit{S. brasiliensis} isolates showed less variability than those observed in \textit{S. schenckii} \textit{s. str.} isolates. These results were consistent with the phylogenetic data [6,9]; the variability among isolates within the species was less frequent in \textit{S. brasiliensis} than in \textit{S. schenckii} \textit{s. str.} No correlation was
found between the electrophoretic karyotype profiles and the cluster organizations among the isolates described in this study.

Variations in chromosome length are common in fungal strains, although the mechanism underlying chromosome length polymorphisms remains unclear. Potential mechanisms include a recombination between repeated sequences that are in the same orientation (which can cause deletions); chromosome breakage and repair (which can lead to translocations and/or deletions); heterochromosomal recombinations; recombinations between subtelomeric regions; and the loss of a chromosome that contains sequences that are not required for growth [40,41]. Recently, chromosome fusion was observed in offspring of the fungus Zymoseptoria tritici, and the probable mechanism was attributed to a breakage-fusion-bridge cycle [42]. Chromosomal length polymorphisms are observed in both asexual and sexual fungal species. For example, in Didymella rabiei, the teleomorphic state of the phytopathogenic fungus Ascochyta rabiei, the karyotype polymorphism was observed among different species isolated across the globe; nevertheless, compared to asexual ascomycetes, A. rabiei had a relatively conserved karyotype [43]. This finding showed that sexual or asexual reproduction was independent of the degree of karyotype polymorphism within a species.

As expected, the present study revealed that the S. schenckii complex displayed extensive polymorphisms in chromosome number and size. The first report of polymorphisms in S. schenckii s.l. showed that isolates from a restricted area of Japan had 6 to 8 chromosomal bands, ranging from 0.5 to 6.2 Mb [10]. In the present study, we found no chromosomal bands smaller than 1.2 Mb; however, we identified chromosomal bands larger than 6.2 Mb. Most of the chromosomal bands observed in both studies were similar in size; however, in our study, polymorphisms in the number and sizes of chromosomal bands were evident. In the present study, we found that the S. schenckii s. str. isolate from Japan had 6 chromosomal bands, and the band sizes were similar to those found by Tateishi et al. [18]. These results showed that, despite different methodologies, our estimations of chromosomal bands sizes agreed with those reported previously. This agreement reinforced our finding that the S. schenckii complex had a high level of chromosome polymorphisms.

The electrophoretic karyotype profiles of isolates showed no correlation with the clinical characteristics or the sources of isolation. This was also described when other techniques were used, such as RAPD. In this study no correlation could be observed among the Brazilian isolates of S. schenckii s.l. from different geographic regions or among the clinical characteristics of sporotrichosis [44]. In Panzincidaceus brasiliensis, Feitosa et al. [21] compared isolates from different sources (human, animal, and soil), from different geographical regions (Brazil, Argentina, Colombia, and Peru), and with different clinical characteristics. They found no correlation between karyotype profiles and clinical/epidemiological characteristics. Canteros et al. [45] also found no correlation between the electrophoretic karyotype and geographic region in Histoplasma capsulatum isolated from Latin American patients. Thus, our results were consistent with those on other thermomorphogenic fungi. The finding that chromosome rearrangements occurred suggested that they may play an important role in the structure of the genome.

Our results showed that the organization of the S. schenckii genome was relatively conserved among different isolates. The variations observed among the isolates suggested the occurrence of chromosome rearrangements, such as translocations. Some of the chromosome rearrangements and/or duplications observed in our study may also have occurred in P. brasiliensis [21]. The hybridization patterns observed with different probes suggested the presence of gene linkage groups and synteny among the S. schenckii species analyzed. The synteny was conserved in closely related species, consistent with observations in other fungi, like P. brasiliensis, where genes were linked on the same chromosome among different isolates [21,46].

The finding that the 7.0 Mb chromosomal band from isolate Ss126 hybridized to multiple bands from the Brazilian isolates suggested the presence of repeated sequences in S. schenckii s. str. and S. brasiliensis isolates. The repeated sequences might reflect the chromosomal rearrangements observed in this study, because repeated sequences can provide a substrate for recombination, duplication, deletion, and inversion events [47]. It is interesting to note that, in S. brasiliensis, the strongest signals were observed in the small-sized bands, and the opposite occurred in S. schenckii s. str. Transposons (one class of repeated sequences) constituted 8–9% of the P. brasiliensis genome and 16% of the P. lutzii genome. Transposable elements were responsible for the addition of 3.0 Mb to the genome of P. lutzii [46]. These results suggested that the differences in chromosome sizes among S. schenckii complex isolates may be related to the number of repetitive sequences.

The minimum haploid genome size was calculated by summing the sizes of the chromosomal bands. For S. schenckii s. str., we calculated an average minimum size of 29.3 Mb, similar to the
isolated from distinct regions of Brazil showed various S. schenckii highly virulent, displayed similar karyotypes. For example, the extremely virulent system [14].

Of an underlying disease that compromised the host immune patient. One potential explanation for the disease was the presence Ss40) [14]. Interestingly, despite the fact that isolate Ss39 (Ss143 and Ss39), medium virulence (Ss141 and Ss16), low virulence (Ss51 and Ss126) and high virulence (Ss40) [14]. Interestingly, despite the fact that isolate Ss39 (S. schenckii) was considered non-virulent, it was isolated from a human patient. One potential explanation for the disease was the presence of an underlying disease that compromised the host immune system [14].

A comparison between karyotype and virulence profiles showed that isolates with different degrees of virulence sometimes displayed similar karyotypes. For example, the extremely virulent S. brasiliensis isolates, CBS 120339 and IPEC 16919 [12], the highly virulent, S. schenckii isolate, Ss126, and the moderately virulent isolate, Ss51 [14], all had a similar profile of 3 large bands and 2 small bands. In contrast, the S. schenckii isolate with low virulence (Ss141) had 3 large bands, 2 intermediate bands, and one small band. Isolates with no virulence had different chromosomal band profiles. For example, Ss39 (S. schenckii) had 7 bands (4 large bands, 1 intermediate band, and 2 small bands) and isolate CBS 120341 (S. mexicana) had only four chromosomes (2 large bands and 2 intermediate bands).

Small chromosomes that carried virulence factor genes were described in Alternaria alternata, a phytopathogenic fungus. Strains of A. alternata pathotypes showed small chromosomes (<1.7 Mb), but these were not observed in nonpathogenic isolates [49]. The small chromosomes that we observed varied from 1.2 Mb to 3.5 Mb. Our results suggested that small chromosomes might carry virulence factors, because the nonpathogenic species, S. mexicana (CBS 120341) did not harbor any small chromosomes, and it was considered non/slightly virulent in other studies [12]. However, the mechanism of pathogenicity appears to be more complicated than simply the presence or absence of small chromosomes as evidenced by the isolate, Ss39, which carried small chromosomes, but the virulence level was distinct from the other isolates in a murine model.

The test for susceptibility to antifungal drugs [15] showed that S. schenckii s. str. and S. brasiliensis were highly susceptible to most antifungals tested in vitro; however terbinafine, ketoconazole, and posaconazole were the most effective drugs. On the other hand, S. globsa and S. mexicana were drug-resistant [15]. In Candida albicans, resistance to fluconazole was associated with chromosome loss and/or gain. In vitro experiments showed that aneuploidy could occur after a week of exposure to fluconazole [50,51]. Also, in C. neoformans, an adaptive mechanism of resistance against azoles, called “heteroresistance”, was found to be due toazole-associated acquisition of aneuploidy after undergoing azole maintenance therapy [52]. It is unknown whether this mechanism might play some role in members of the Sporothrix schenckii complex, but isolates tested in previous studies were resistant to fluconazole [15].

Our results suggested that aneuploidy could occur in Sporothrix, and it is tempting to hypothesize that this phenomenon could be responsible for the differences in drug resistance, as described for other fungi [52]. Genome-wide mapping of fungal chromosomal bands would be a valuable future project that could elucidate the role of chromosomal polymorphisms in the biology of the S. schenckii complex.

Conclusions

In light of recent taxonomic changes in the genus Sporothrix, this study was the first to analyze chromosomal polymorphisms in diverse isolates of the S. schenckii complex and to assign genes to chromosomal bands of S. schenckii s. str. and S. brasiliensis isolated from Brazil. The minimum genome size of S. schenckii complex isolates was variable. Based on data from genome sequencing, we hypothesized that the isolates studied here may have harbored two 7.0 Mb chromosomes. The virulence in Sporothrix spp. is a complex phenomenon that may be orchestrated by polymorphisms in chromosomes, and deserved to be studied further. Overall, our results provided the number of chromosomes in these fungi; this information will facilitate the complete assembly of the genome sequences, and it will contribute to the understanding of the genomic organization of these species.

Supporting Information

Figure S1 Densitometric analysis of S. schenckii isolates from Brazil. Each panel shows (left) the ethidium bromide-stained gel after pulsed field gel electrophoresis of chromosomes from the fungus strain indicated, and (right) a graph of the densitometric analysis. The size of each chromosomal band (Mb) is indicated on the left and above the corresponding peaks on the graph. Open arrows indicate where samples were loaded.
The Figure S2 densitometric analysis of S. schenckii from Brazil. Each panel shows the ethidium bromide-stained gel after pulsed field gel electrophoresis of chromosomes from the fungus strain indicated, and a graphic of the densitometric analysis. The size of each chromosomal band (Mb) is indicated on the left and above the corresponding peaks on the graph. Open arrows indicate where samples were loaded.

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**Table S1:** Genes, primer sequences, PCR programs, and references used in this study. (DOCX)

**Author Contributions**

Conceived and designed the experiments: AAS LFdB FM MM. Performed the experiments: AAS. Analyzed the data: AAS GFF AMR FML MM JFB. Contributed reagents/materials/analysis tools: MdMT MSZ PC. Wrote the paper: AAS LFdB FML MM ZPdC.
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