Brazilian Journal of Microbiology (2021) 52:91–100
https://doi.org/10.1007/s42770-020-00346-6

CLINICAL MICROBIOLOGY - RESEARCH PAPER

Phenotypic and molecular characterisation of *Sporothrix globosa* of diverse origin from India

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Received: 17 December 2019 / Accepted: 21 July 2020 / Published online: 30 July 2020

Abstract

Sporotrichosis is one of the neglected tropical diseases causing subcutaneous chronic granulomatous lesion by thermally dimorphic fungi belonging to *Sporothrix* species. *Sporothrix brasiliensis*, *Sporothrix mexicana* and *Sporothrix globosa* are the common pathogenic species. In Asian countries, *S. globosa* constitutes nearly 99.3% of all *Sporothrix* species. We studied 63 cases of sporotrichosis of geographically diverse origin from India and *Sporothrix* isolates were characterised for its growth in different media, temperatures, ability to assimilate sugars and antifungal susceptibility profile. Molecular characterization was performed by sequencing of the calmodulin (CAL), beta tubulin (BT) and translational elongation factor 1-alpha (TEF-1α) and typing by fluorescent amplified fragment length polymorphism (FAFLP). In patients who presented with fixed (49.2%), lymphocutaneous lesions (23.8%), in 26.9% the details were not known, none had systemic dissemination. All the isolates tested were *Sporothrix globosa* and that could grow up to 35 °C and unable to grow at and beyond 37 °C. The assimilation of sucrose, ribitol and raffinose helps in identifying *S. globosa*. Sequences of CAL or BT or TEF-1α can differentiate *S. globosa* from other species in the complex. FAFLP results exhibited low genetic diversity. No correlation was noted between genotypes and clinical presentation, or geographic distribution. Itraconazole, terbinafine and posaconazole showed good in vitro antifungal activity against *S. globosa* whereas fluconazole and micafungin had no activity. *S. globosa* of Indian origin is relatively less pathogenic than other pathogenic *Sporothrix* species as it does not cause systemic dissemination and in the diagnostic laboratory, incubation of the cultures below 37 °C is essential for effective isolation.

Keywords Sporotrichosis · *Sporothrix globosa* · Dimorphic fungi · Genetic typing · Antifungal · Calmodulin · Taxonomy

Introduction

Sporotrichosis is a chronic granulomatous subcutaneous mycoses caused by different thermally dimorphic *Sporothrix* species [1]. In recent years, this dimorphic fungus has received special interest due to an increased number of infections causing epidemics and outbreaks [2]. Several phylogenetically related *Sporothrix* species are cosmopolitan in nature and are

Responsible Editor: Sandro Rogerio de Almeida.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s42770-020-00346-6) contains supplementary material, which is available to authorized users.

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commonly found in tropical and subtropical countries [1, 3–6]. Humans acquire the infection by accidental percutaneous inoculation of the fungus present on a variety of vegetation, decomposing wood or soil, or by zoonotic transmission [2, 7]. Rarely, the respiratory route is the mode of acquisition [8]. Occupation-dependent infections are most frequent in the Indian subcontinent, unlike epidemics in Latin American countries where zoonotic transmission is the commonest mode of transmission [9, 10]. The severity of the disease varies from fixed cutaneous plaques/ulcerative lesions to lymphocutaneous spread to dissemination in immunocompromised patients [11, 12]. In India, the first case was reported from Calcutta in 1932 [13]. In later years, cases of sporotrichosis were reported from all over the country [7, 14–18]. As per the recent survey, it has been identified that the laboratory diagnosis of sporotrichosis is the most problematic with poor sensitivity [19].

Molecular studies from different geographical regions revealed the presence of many cryptic species in *Sporothrix schenckii* based on the partial calmodulin gene [20–22]. Marimon et al., proposed the existence of three pathogenic species, *Sporothrix brasiliensis*, *Sporothrix mexicana* and *Sporothrix globosa* within the *S. schenckii* complex, which were geographically distinct. In Asian countries, *S. globosa* is endemic and constitutes nearly 99.3% of all *Sporothrix* species [23]. All the Indian strains tested belonged to *S. globosa* [20]. Besides Asian countries, *S. globosa* has also been reported in Europe, the Americas and Australia [6, 23]. *S. globosa*, has a low degree of genetic variation in contrast to *S. schenckii* and *S. brasiliensis* [23, 24]. Recently, *Sporothrix mexicana*, *Sporothrix pallida* and *Sporothrix chilensis* were reported to cause human infections [23, 25, 26].

S. globosa is fourth in the virulence hierarchy after *S. brasiliensis*, *S. schenckii*, *S. luriei*, [27–30]. Several reports suggest variations in susceptibility profiles for *Sporothrix* species [31–33]. Sporotrichosis is treated by saturated potassium iodide or itraconazole for cutaneous and lymphocutaneaous forms, and amphotericin B for disseminated or itraconazole in treatment failure cases [34]. With the growing reports of treatment failure in *S. globosa* infections [35], routine antifungal susceptibility testing of the isolates becomes necessary.

Diversity in the genome, antifungal susceptibility, virulence of the isolates along with geographical distribution has made necessary to identify *Sporothrix* to species level for proper patient management. The present study is, therefore, carried out to determine the distribution and genetic diversity of *Sporothrix* species isolated across India and also to determine the antifungal susceptibility profiles of these isolates.

Materials and methods

Strains A total of 63 isolates preserved as *Sporothrix schenckii* sensu lato at the National Collection Centre for Pathogenic Fungi (NCCPF), Postgraduate Institute of Medical Education and Research, Chandigarh, India, were included in the study. All the isolates were from human clinical samples and were isolated from geographically diverse places across India. Demographic details such as age, sex, type of lesions, year of isolation and geographic origin associated with each case were noted. The isolates were revived from the lyophilised vials and sub-cultured on potato dextrose agar (PDA) slants before using for the morphological, physiological and molecular studies.

Morphological studies Colony morphology of all the isolates was studied on PDA, cornmeal and oatmeal agar at different temperatures (25, 30 and 35 °C). For the transition of mycelial to yeast phase, the cultures were inoculated on to brain heart infusion agar with sheep blood agar (BHIBA) incubated at 35 °C for up to 21 days. The colonies on BHIBA were suspended in saline to check for yeast conversion. To study the microscopic features of the mycelial phase, the isolates were subjected to micro-slide cultures on cornmeal agar. After incubation at 30 °C for 14 or 21 days, the coverslips were removed from the slide culture, stained with lactophenol cotton blue and examined under a light microscope.

Physiological studies To determine the growth rate at various temperatures (25, 30, 35 and 37 °C), each isolate was inoculated with a 1-mm square piece of fungal growth (on PDA) on to the duplicate fresh PDA plates. The colonies were measured after 14 days and the mean value was recorded. The capability to assimilate sucrose, ribitol and raffinose was performed in liquid nitrogen base media in 96-well microplates as per the method described by Yarrow [36].

DNA extraction, amplification and sequencing Sequencing reaction/molecular identification using primers to amplify CAL, BT and TEF1-α was carried out for all the 63 isolates. Degenerate primers CL1 (5′-GARTWCAAGGAGG CCTTCTC-3′) and CL2A (51-TTTTTGCATCATGA GTTGGAC-3′) were used to amplify the CAL locus region as described by O'Donnell [37]. Bt2-F [5′ GG[CT]AACCA(AG)AT(ATC)GGTGC(CT)GC(CT)3′] and Bt2-R [5′ACCCTC(AG)GTGTAGTGACCCTTGGC3′] were used to amplify beta tubulin, and EF1-F (5′-CTGA GGCTCGTTACCAGGAG-3′) and EF1-R (5′-CGAC TTGATGACACCGACAG-3′) primers were used to amplify TEF1-α region. Briefly, isolates were cultured on to PDA and incubated at 25 °C for 1 week, and DNA extraction and purification was carried out using a phenol chloroform extraction method. DNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, USA). The amplification of all the three gene (CAL, BT and TEF1-α) fragments was carried out directly from genomic DNA. For a reaction volume of 25 μl, 2.5 μl of the 1:10

diluted genomic DNA and 0.5 mM of each primer were added. PCR products were amplified with the following temperature profiles: denaturation for 5 min at 94 °C, followed by 35 cycles of 30 s at 95 °C, 1 min at the annealing temperature of 60 °C (CAL and BT) and 57 °C (TEF1-α) and 1 min at 72 °C, followed by final extension for 5 min at 72 °C. Sequencing PCR was carried out using Big Dye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA, USA) as per the kit protocol. For each gene fragment, both the positive strand and the negative strand were sequenced using both forward and reverse primer respectively. The sequence analysis was carried out with ABI 3130 genetic analyser (Applied Biosystems). The sequences of all the strains were deposited in GenBank under the accession numbers as mentioned in supplementary table – 1.

All the three gene sequences of the isolates were verified by BLASTN (http://www.ncbi.nlm.nih.gov/blast) search analysis. Furthermore, forward and reverse sequences of respective strains and genes were assembled into single contig via Lasergene's Seqman software. Mis-paired base pairs were manually edited in the consensus sequence.

Phylogenetic analysis Sequences of CAL, BT and TEF1-α of *S. schenckii* species of clinical importance belonging to the different clades were retrieved from GenBank. Multiple sequence comparison was performed using Clustal X2 algorithm and implemented in MEGA 6 software. *Grosmannia serpens* (CBS 141.36) [38], a saprophytic fungus belonging to *Ophiostomataceae*, was used as an outgroup for CAL analysis [39]. For the BT and TEF1-α analysis *Ophiostoma piliferum* (CBS 158.74) was used as outgroup [40].

Phylogenetic analyses were carried out using neighbour-joining method. Neighbour-joining trees were constructed using MEGA 6 software [41] and 1000 bootstrap replicates were used to estimate confidence values for individual clades [42]. The evolutionary distances were computed using the Kimura 2-parameter method [43]. Genetic diversity and haplotyping of CAL, BT and TEF1-α genes were estimated using DNAsp v5.10 [44]. Gaps and missing data were excluded in the calculation and the rate variation among sites was modelled with a gamma distribution (shape parameter = 1).

Antifungal susceptibility testing Minimum inhibitory concentrations (MIC) were determined for antifungal agents' fluconazole, itraconazole, voriconazole, posaconazole, amphotericin B, terbinafine and micafungin (Sigma-Aldrich, India) against 63 *Sporothrix* isolates. Testing was carried out by the microbroth dilution technique according to the Clinical and Laboratory Standards Institute's M38-A2 method [45]. The final drug concentration was adjusted from 0.03 to 16 μg/ml for fluconazole, itraconazole, voriconazole, posaconazole, amphotericin B and terbinafine, 0.03 to 4 μg/ml for micafungin. The *Sporothrix* isolates were freshly grown on PDA for 5–7 days at 25 °C; the conidia were harvested and used to prepare inoculum. Inoculum size was established at an optical density ranging 0.09 to 0.13 in a spectrophotometer at 530 nm. Inoculated plates were incubated at 35 °C and results were observed visually after 3 days. For quality control, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 strains were used.

Fluorescent amplified fragment length polymorphism genotyping *Sporothrix* isolates were subjected to genotyping by fluorescent amplified fragment length polymorphism technique as previously described by Chakrabarti et al. [46]. Briefly combined restriction and ligation step was carried out using *EcoRI* and *MseI* enzymes and T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). Amplification with pre-selective primers *EcoRI* (5′GACTGCGTACCAATTC-3′) and *MseI* (5′GATGAGTCCTGAGTAA-3′) was performed. *EcoRI* with one selective residue (5′-Flu-GACTGCGTACCAATTCAC-3′) and *MseI* primer with two selective residues were used (5′-GATGAGTCCTGAGTAACG-3′) for selective amplification. One of the selective primers labelled with 6-FAM. LIZ-500 was used as a standard marker. Capillary electrophoresis of the amplified products was carried out in ABI-automated DNA sequencer 3130 (Applied Biosystems, Foster City, CA, USA). Typing data were imported to BIONUMERICS v7.6 software (Applied Maths, Ghent, Belgium). Fingerprint curves were converted into bands and correct bands of each lane were assigned using the band position of the reference dye (LIZ500). The similarity coefficients were determined by the Pearson correlation with negative similarities clip to zero. Cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using BIONUMERICS software.

Results

Among the 63 culture-proven sporotrichosis cases evaluated, 31 patients had a history of fixed cutaneous type and 15 patients had a lymphocutaneous type of infection whereas clinical details of 17 patients were not available. The majority of the patients were in the age group of 32 to 60 (mean age—46.7 years). The oldest patient was an 85-year-old female and the youngest patient was a 15-year-old male. Of the 44 cases with available demographic details, 21 were males and 23 females. The 63 clinical isolates were received from different geographical areas of India. Forty-seven isolates were from patients of northern India (24 from Himachal Pradesh, 21—Chandigarh and 2—Delhi), 6 isolates were from eastern India (3—Kolkata, 3—Assam) and 10 isolates were from southern India (8—Belgaum and 2—Bengaluru) (Table 1).

Table 1 Demographics, clinical type, isolation place and year of *Sporothrix* species included in the study

Serial. No	NCCPF No	Age/sex	Lesion type	Year	Place of origin
1	220007	50/F	Lymphocutaneous	1994	Chandigarh
2	220010	30/M	Fixed	1995	Chandigarh
3	220011	22/M	Fixed	1995	Chandigarh
4	220012	35/M	Lymphocutaneous	1996	Belgaum
5	220026	36/F	Fixed	1996	Chandigarh
6	7220027	85/F	Fixed	1996	Chandigarh
7	220028	37/M	Fixed	1996	Belgaum
8	220029	45/F	Fixed	1996	Kolkata
9	220030	60/F	Lymphocutaneous	1996	Chandigarh
10	220032	65/F	Fixed	1997	Chandigarh
11	220037	NA	NA	1997	Kolkata
12	220038	48/M	Lymphocutaneous	1997	Belgaum
13	220040	53/F	Fixed	1997	Chandigarh
14	220041	65/M	Lymphocutaneous	1997	Chandigarh
15	220045	35/F	Fixed	1997	Kolkata
16	220047	46/M	Fixed	1997	Chandigarh
17	220048	55/M	Lymphocutaneous	1996	Belgaum
18	220049	55/F	Lymphocutaneous	1997	Himachal Pradesh
19	220071	38/F	Fixed	2000	Chandigarh
20	220072	NA	NA	2001	Chandigarh
21	220073	NA	NA	2001	Chandigarh
22	220076	35/M	Lymphocutaneous	2001	Belgaum
23	220078	72/M	Lymphocutaneous	2002	Chandigarh
24	220079	44/F	Fixed	2002	Himachal Pradesh
25	220080	32/M	Fixed	2003	Chandigarh
26	220082	36/F	Lymphocutaneous	2003	Belgaum
27	220087	44/M	Lymphocutaneous	2005	Chandigarh
28	220088	15/M	Lymphocutaneous	2006	Chandigarh
29	220089	NA	NA	2006	Chandigarh
30	220090	65/F	Fixed	2006	Chandigarh
31	220091	72/M	Lymphocutaneous	2006	Himachal
32	220093	58/F	Fixed	2007	Chandigarh
33	220094	42/M	Lymphocutaneous	2008	Himachal Pradesh
34	220095	36/M	Fixed	2008	Bangalore
35	220101	NA	Fixed	2010	Delhi
36	220103	NA	Fixed	2010	Delhi
37	220105	NA	NA	2010	Bangalore
38	220111	NA	Lymphocutaneous	2011	Himachal Pradesh
39	220112	NA	NA	2011	Himachal Pradesh
40	220113	NA	NA	2011	Himachal Pradesh
41	220114	NA	NA	2011	Himachal Pradesh
42	220116	NA	NA	2012	Himachal Pradesh
43	220118	NA	NA	2012	Himachal Pradesh
44	220119	NA	NA	2012	Himachal Pradesh
45	220120	NA	NA	2012	Himachal Pradesh
46	220122	NA	NA	2012	Himachal Pradesh
47	220124	NA	NA	2012	Himachal Pradesh
48	220125	25/F	Fixed	2012	Himachal Pradesh
49	220126	45/F	Fixed	2013	Belgaum
50	220127	45/F	Fixed	2013	Belgaum
51	220129	57/F	Fixed	2015	Chandigarh
52	220135	NA	Fixed	2015	Assam
53	220136	NA	NA	2015	Assam
54	220137	NA	NA	2015	Assam
55	220138	56/M	Fixed	2015	Himachal Pradesh
56	220139	30/M	Fixed	2015	Himachal Pradesh
57	220240	40/F	Fixed	2015	Himachal Pradesh
58	220142	26/F	Fixed	2015	Himachal Pradesh
59	220143	63/M	Fixed	2015	Himachal Pradesh
60	220144	55/F	Fixed	2015	Himachal Pradesh
61	220145	62/M	Fixed	2015	Himachal Pradesh
62	220146	48/F	Fixed	2015	Himachal Pradesh
63	220149	NA	NA	2015	Himachal Pradesh

Colony characteristics on three different media tested were similar with no significant variation. After 21 days of incubation at 30 °C, the colonies were grey which later produced brownish to dark brown–coloured colonies. The colony diameter measured between 18 and 26 mm (mean diameter = 21.8 mm) on plates incubated at 25 and 30 °C, whereas colony diameter measured from 6 to 24 mm (mean diameter = 14.2 mm) on plates incubated at 35 °C. On CMA and oatmeal agar, colonies of 63 isolates were brown to dark brown at similar incubation conditions. Colonies on BHIBA appeared white to light brown yeast-like growth after 10–12 days of incubation. Failure to convert to the yeast phase was noted in approximately half of the isolates tested (15/29). Microscopically, the yeasts are elongated or cigar-shaped measuring about 5–7 μm × 1–3 μm.

The isolates produced conidia in clusters either terminal or intercalary on well-differentiated conidiophores. Conidiophores were swollen and produced sympodial conidia on the denticles. The conidia were hyaline to subhyaline, obovoidal measuring about 3–5 μm × 1–3 μm. In addition, the second type of conidia described as sessile conidia was produced within 14 days in most of the isolates and by day 21 in all isolates. The sessile conidia were brown to dark brown, thick walled, globose to subglobose. All the isolates assimilated sucrose and ribitol whereas raffinose was negative for all isolates.

The sequences 733, 395 and 708 nucleotides of CAL, BT and TEF-1α respectively were amplified and all the isolates were identified as *S. globosa*. The phylogenetic tree constructed based on CAL, BT and TEF-1α is presented in supplementary figure 1-3 respectively. The combined dataset (CAL + TEF-1α + BT) yielded a sequence alignment of 1836 positions, including 1807 invariable characters, 27 (1.5%) variable parsimony informative sites and 10 singleton variable sites. The concatenated sequences of CAL + BT + TEF-1α yielded 23 haplotypes on haplotyping analysis. Haplotype diversity (Hd) and nucleotide diversity (Pi) were 0.906 and 0.00245 respectively.

Genotyping analysis of the amplified fragment of three individual gene loci was performed using DNAsp software 5.10. The aligned CAL sequences were 733 bp long, including 717 invariable sites with 12 parsimony informative sites. The haplotype analysis of the CAL sequence divided the isolates into 10 Hap groups (Hd = 0.6114) (Pi = 0.00322). The aligned BT sequences were 395 bp long, including 391 invariable sites with 2 parsimony informative site and 4 singleton variable sites. The haplotype analysis of BT sequences divided the isolates into 6 Hap groups (Hd = 0.661) (pi = 0.00265). The aligned TEF1-α sequences were 708 bp long including 699 invariable sites with 3 parsimony informative sites and 6 singleton variable sites. The haplotype analysis of the TEF1-α sequence divided the isolates into 10 Hap groups. All the haplotypes (Hd = 0.7035) belonged to the same group with less nucleotide diversity (Pi = 0.00270) (Table 2).

A total of 55 isolates yielding sufficient bands were included in the FAFLP (fluorescent amplified fragment length polymorphism) analysis. FAFLP profiles yielded fragments ranging from 10 to 390 base pairs, but only fragments ranging from 25 to 300 bp were included for the analysis. All *Sporothrix* isolates had more than 60% similarity. FAFLP profile yielded 12 clusters (Fig. 1). The majority of the isolates formed three major clusters, i.e. cluster 'A', 'E' and 'F'. In the cluster 'A', all the isolates belonged to northern India except for one isolate. Cluster 'E' comprised most of southern India isolates. Cluster 'C', 'D', 'G', 'I', 'J' and 'L' consisted of single isolate whereas 'B', 'H' and 'K' had two isolates each.

The MIC, geometric mean, MIC50 and MIC90 for tested isolates against various antifungals are provided in Table 3.

Discussion

The present study describes the clinical features of sporotrichosis cases across India and characterises the isolates from those cases, both phenotypic and molecular characters, and antifungal susceptibility profile. The majority patients were males, but there was no significant difference between male and female patients similar to the report of China [47]. However, our study was only on referred cases to our centre. So no definite demographic specifics can be made from referred cases. Earlier studies from north India indicated females, who were involved in agricultural activities, were common sufferer [10, 14]. A meta-analysis by Zhang et al., in 2015 reported that *S. globosa* was common in the female population in different continents [23]. A study from northeast China also reported female preponderance and their involvement in agricultural activities. Sporotrichosis can affect persons regardless of age and gender. Occupational and other recreational activities influence the rate of infection in different population [48].

Phenotypic characterization of our *Sporothrix* isolates showed maximum growth (mean growth rate 21.8–21.9 mm) on PDA media incubated at 25 °C and 30 °C after 21 days of incubation, but stunted growth (mean 14.2 mm) at 35 °C and no growth at 37 °C. The findings are in concordance with the study by Marimon et al., in which none of the *S. globosa* isolates tested grew at 37 °C [49]. In contrast to our findings, *S. globosa* isolates of Venezuelan origin grew at 37 °C after 21 days of incubation [50] and 97% of the isolates from China exhibited stunted growth at 37 °C [51]. According to Dixon et al., *Sporothrix* isolates that produce pigmented conidia can grow at 37 °C and are virulent in the murine model [52]. Kwon-Chung et al., reported that the isolates obtained from lymphocutaneous lesions were able to grow at 37 °C unlike isolates from fixed cutaneous which exhibited growth at 35 °C [53]. However, our isolates irrespective of origin from lymphocutaneous or fixed cutaneous lesion produced

Table 2 Comparison of nucleotide and haplotype variations among calmodulin, beta-tubulin and Translational elongation factor 1-α genes

Parameter	Calmodulin	Beta-tubulin	Translational elongation factor 1-α
S (number of variable sites)	14	4	9
H (total no. of mutations)	14	4	9
π (nucleotide diversity per site)	0.00322	0.00265	0.00270
h (number of haplotypes)	10	6	10
h_d Haplotype diversity	0.611	0.661	0.7035
k average number of nucleotide differences	2.344	1.041	1.111

dark brown pigmented conidia after 21 days of incubation but did not grow at 37 °C. The above phenotypic characters described by several workers were before the description of the new *Sporothrix* spp. It is now believed that the differences in phenotypic characters may be attributed to the different *Sporothrix* species implicated rather than the type of lesion and geographical location of the isolates. The report from northeast China noted *S. globosa* was responsible for all clinical types of sporotrichosis [54]. Generally, *S. globosa* isolates from all geographical regions are less virulent compared with *S. schenckii* sensu stricto (25,54,55).

Morphologically, *Sporothrix* species varies minimally and it is difficult even for the experienced mycologist to differentiate different species within the genus *Sporothrix*. Marimon et al., after testing a large panel of sugars, reported that assimilation of sucrose, ribitol and raffinose can help in the identification of *S. globosa* [20]. Hence, sugar assimilation can be successfully used in the resource constrained laboratory for the preliminary identification of *S. globosa*. In the present study, all isolates were ribitol and sucrose assimilation positive and raffinose negative. In the study of Marimon et al., 91% of *S. globosa* isolates assimilated ribitol. Possibly, such studies with more isolates from the different geographical region are required for confident identification of *Sporothrix* species phenotypically.

Though *S. globosa* is considered as a cosmopolitan species, it exhibits geographical preferences [55] with prevalence rate highest in Asia (56%) and least in Africa (5%) whereas in Europe and Americas, its prevalence is 28% and 11% respectively [55]. In this study using multigene sequencing, we confirmed that all the *Sporothrix* isolates obtained from various parts of India are *S. globosa*. Based on phylogenetic analysis, Indian isolates along with isolates of China, Japan, Spain, Italy and the USA were in the clade III clinical group (*S. globosa*) [20]. The majority of the *Sporothrix* species prevailing in Asia belong to *S. globosa* (99.3%) [23]. Instead of three genes, the six species of clinically important *Sporothrix* species can be differentiated by the CAL sequence only [20]. In this study, the calmodulin gene also identified all the Indian isolates as *S. globosa* (Supplementary Fig. 1) and it is the only causative species of sporotrichosis in India. Until the recent report of the presence of *S. schenckii* in the central part of China, it was thought that only *S. globosa* existed in that country [47, 54, 56]. According to Yu et al., *S. globosa* isolates from the Northeastern part of China differentiated into two subclades. Majority of the isolates in our study grouped under subclade I and only six isolates grouped under subclade II along with Italian isolate [54, 57]. In the present study, 16 isolates grouped along with the representative strain from subclade II of Chinese and Italian isolates. Phylogenetic analysis revealed that our isolates are genetically similar to environmental isolates of China and clinical isolates of Spain, Japan, Columbia and the USA (Supplementary Figure 1). This finding corroborates with other reports where *S. globosa* isolates originating from different continents displayed identical profile or low genetic diversity [23, 24, 58]. Gong et al., recently reported the utility of microsatellite typing in identifying genetically diverse *S. globosa*, similar to a multigene approach (*CAL*, TEF1-α or *BT* genes) [59]. However, whole genome sequencing results from Australian *S. globosa* isolates revealed low genome diversity [6].

Haplotyping analyses placed all our isolates into 10 haplotypes whereas *S. globosa* isolates belonging to Venezuela had only five haplotypes [50]. High genetic variation was observed in *S. brasiliensis* and *S. schenckii* sensu stricto [58]. Similar findings were also reported by Moussa et al., in which the *S. globosa* population had low diversity (9 haplotypes) compared with *S. schenckii* (42 haplotypes). The meta-analysis by Zhang et al., also showed that in defined endemic areas, a single molecular type of *S. globosa* is preponderant (> 80%) [23].

BT and TEF1-α were also used as markers to assess the genetic diversity within the species. Phylogenetically BT sequence can also differentiate *S. globosa* from other *Sporothrix* species [55]. This is in contrast with the study from Marimon et al. [20], where the *BT* sequences from representative *Sporothrix* species (*S. brasiliensis* AM116946, *S. globosa* AF116966 and *S. mexicana* AM498344) were in very low homology. Analysis of the BT sequence grouped our isolates into three clades (Supplementary Figure 2). But all our isolates are grouped together along with the *S. globosa* standard strain (AM116966). Intraspecific genetic variation is higher in the TEF1-α gene among *S. globosa* isolates compared with *S. brasiliensis*, where haplotyping revealed only three

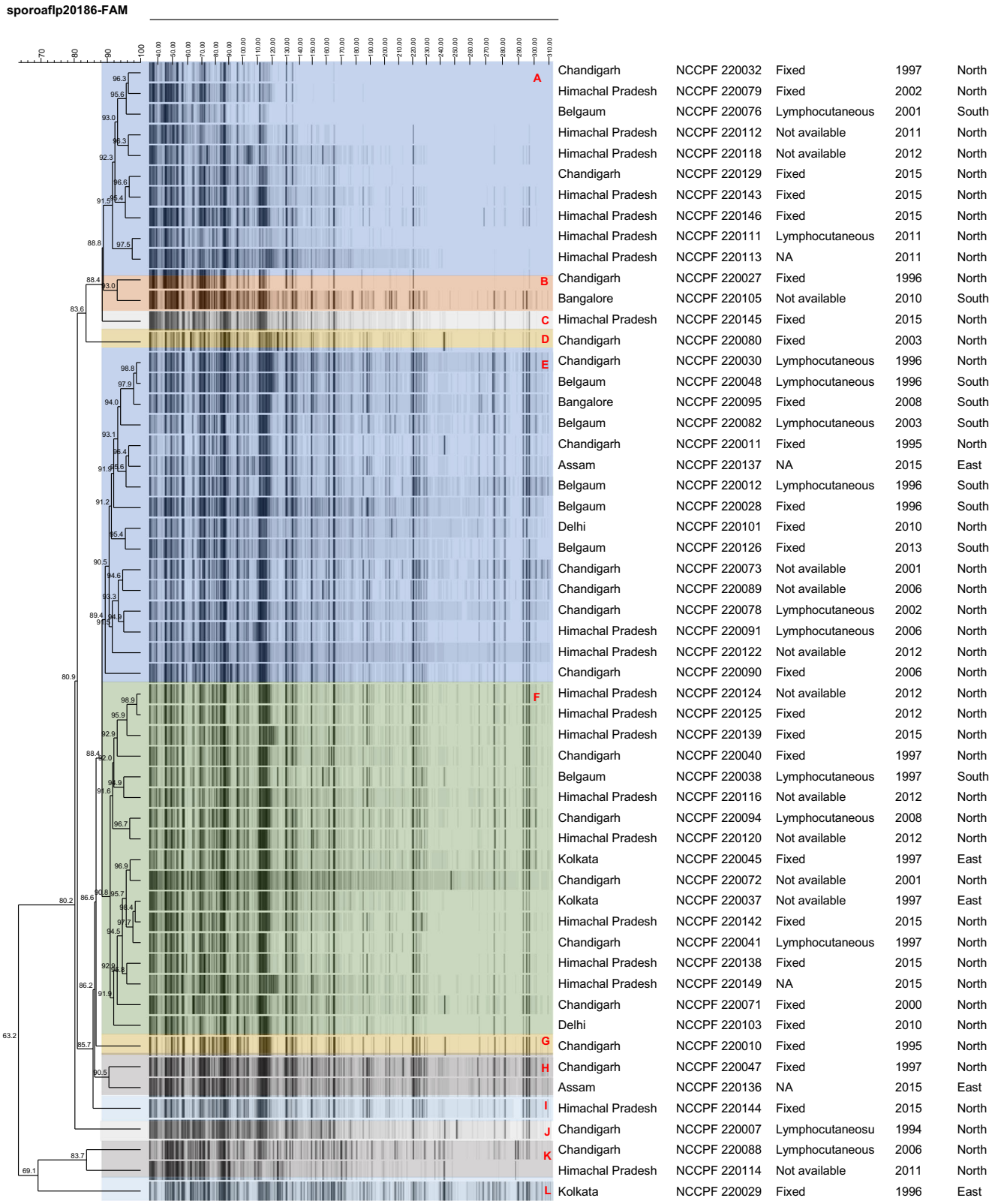

Fig. 1 Dendrogram generated by UPGMA analysis of FAFLP profiles obtained from *S. globosa*

haplogroups [2]. However, the TEF1-α gene was able to differentiate two genotypes responsible for causing an epidemic in Brazil at the same time period. Haplotyping analysis of TEF1-α gene of our isolates showed 10 different haplotypes, with 31 isolates in type III, 13

Table 3 Antifungal susceptibility profile of *Sporothrix globosa* isolates of Indian origin against 7 antifungal agents (values in μg/ml)

Antifungal agent	Range	MIC50	MIC90	GM
Fluconazole	2–16	16	16	13.27
Itraconazole	0.03–2	0.25	1	0.218
Voriconazole	0.03–16	0.25	8	0.454
Posaconazole	0.03–2	0.25	1	0.315
Amphotericin B	0.03–8	1	2	1.064
Micafungin	4	4	4	4
Terbinafine	0.03–2	0.03	0.06	0.043

isolates in type I, 7 isolates in type IX, 6 isolates in type VII and one isolate each of remaining types.

FAFLP has been shown to be a useful tool to study the molecular epidemiology of *S. globosa*. In a study by Gong et al., microsatellite marker–based typing has shown similar potential as FAFLP in discriminating geographic variation among *S. globosa* [59]. We used FAFLP in the present study as it is relatively cheaper and less cumbersome than microsatellite-based typing. FAFLP analysis divided our strains into 12 different clusters. FAFLP genotypes have not been found to be related to pathology, disease profile or geography whereas FAFLP profiles of *S. globosa* isolates of China exhibited regional difference [51]. The probable reason for multiple genotypes originating from a single region may be due to frequent migration of population across the country. FAFLP analysis by Zhang et al., also showed a low degree of variation among *S. globosa* isolates from different regions compared with other species in the *Sporothrix* species. This is in concordance with the study from Neyra et al., in which all the Peruvian isolates clustered into two groups irrespective of their pathobiology and geography within the area [57]. Low genetic variability among *S. globosa* from different continents having large geographic distance may be due to the emergence of rapid vectors that have a critical role in the dispersal of *S. globosa* across continent. Rangel-Gamboa et al., hypothesise that organic products and the exchange of foods via the international market could be the reason for its worldwide transmission [24]. Similar to previous reports, we also did not find any specific association between the FAFLP genotypes and susceptibility profile [51].

Antifungal susceptibility testing for *Sporothrix* species is performed according to the Clinical Laboratory Standard Institute (CLSI) methods [29, 60–62]. There are no defined breakpoints or epidemiological cut-off values (ECV) available for *S. globosa* [63]. Even reports on the antifungal susceptibility profile of *S. globosa* are scarce. Our results showed that itraconazole is an effective drug with MIC 90 of 1 μg/ml, whereas *S. globosa* isolates from Japan had poor activity against itraconazole compared with isolates tested in this study [64]. Voriconazole exhibited varied in vitro antifungal activity. While the majority (87.3%, 55 isolates) had lower MICs, only eight isolates (12.7%) exhibited higher MICs ranging between 4 and 16 μg/ml. Like other reports, our reports also confirm that fluconazole and micafungin have no activity on *S. globosa* [29, 60, 65]. Terbinafine showed good activity with low MIC values which is consistent with other studies on *S. schenckii* and *S. globosa* [29, 30, 51, 66–68]. But the clinical efficacy of terbinafine for the treatment of sporotrichosis due to *S. globosa* is not known and worth evaluating.

In conclusion, all the *Sporothrix* isolates across different geographic regions of India are *S. globosa.* None of our isolates were from disseminated cases and did not grow at 37 °C. Sucrose, ribitol and raffinose could be employed for differentiating *S. globosa* from other *Sporothrix* species. CAL, BT and TEF1-α genes could be used to differentiate *S. globosa* from other species in the complex. The translational elongation factor gene has more genetic variability compared with other genes. FAFLP analysis yielded a significant number of genotypes but is not associated with phenotypic characters or geographic locations. Itraconazole and terbinafine exhibited good in vitro activity.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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