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Molecular identification of *Sporothrix* clinical isolates in China*

Ting-ting LIU[1], Ke ZHANG[2], Xun ZHOU[†‡1]

([1]*Department of Dermatology, Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China*)

([2]*State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China*)

[†]E-mail: zhouxun123@sina.com

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Abstract: In this study, we investigated the molecular phylogeny of 64 clinical isolates which were identified as *Sporothrix schenckii sensu lato* by morphological identification. All of the strains were isolates from patients from several provinces in China. The phylogeny was inferred by DNA sequence analyses based on datasets of the ribosomal internal transcribed spacer (ITS) and a combined ITS and partial β-tubulin region. Reference sequences were retrieved from GenBank. Results showed that all of the isolates were clustered in a distinct clade with a type of *Sporothrix globosa*. Our analysis showed that *S. globosa* is the causal agent of the tested sporotrichosis in China, rather than *S. schenckii* that was generally believed to be the case. The existence of *S. schenckii* in China remains to be confirmed. This study improved our understanding of the distribution of the species in *S. schenckii* complex.

Key words: Internal transcribed spacer (ITS), Diagnosis, *Sporothrix globosa*, Taxonomy, Phylogeny
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1 Introduction

Sporotrichosis is an acute or chronic mycosis caused by infection of the *Sporothrix schenckii*, and recently is demonstrated to be a species complex (López-Romero *et al*., 2011). Humans and animals acquire it from inoculation of the fungus through thorns, traumas and scratches or bite wounds (de Lima Barros *et al*., 2004; Schubach *et al*., 2008), even the spores can enter through the respiratory tract. Sporotrichosis may develop as a disseminated disease in immunocompromised individuals particularly in acquired immunodeficiency syndrome (AIDS) patients (Yap, 2011). Sporotrichosis has a global distribution. In China, it is more frequently encountered in the northeast provinces including Jilin, Heilongjiang, and Liaoning (Zhang and Lin, 2008; Mei *et al*., 2011; Song *et al*., 2011a; 2011b).

S. schenckii had long been believed to be the unique causal agent of sporotrichosis. Identifications were made primarily based on morphological characters (Lopes-Bezerra *et al*., 2006). Phylogenetic analysis based on the sequences of the internal transcribed spacer (ITS) region suggested that more than one species existed in this morphologically defined group, indicating this is a species complex (de Beer *et al*., 2003). Recently, isolates received as *S. schenckii* were regrouped into at least six cryptic species by multilocus phylogenetic analysis, and exhibited a degree of geographical specificity (Marimon *et al*., 2006; 2007; Madrid *et al*., 2010). Different clinical forms of sporotrichosis are related to the host condition, the route of infection, and the virulence of pathogen, while the virulence was suggested to be associated with different species of *Sporothrix* (Arrillaga-Moncrieff *et al*., 2009; Fernandes *et al*., 2013). Therefore, it is important to recognize what are the real causal agents of the sporotrichosis in various regions, because of the significant differences in the guiding clinical management among these species.

‡ Corresponding author

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Clinical *Sporothrix* species have previously been classified based on the partial calmodulin sequences (Marimon *et al*., 2007; 2008b; Tan *et al*., 2013), but calmodulin regions have been reported to have a lower amplification efficiency as compared to more commonly used regions such as the ribosomal ITS and β-tubulin. ITS is widely used in fungal identification, and recommended as the general barcode for fungi, although additional markers might be needed to distinguish species in certain complexes (Schoch *et al*., 2012). Zhou *et al*. (2013) concluded that all the *Sporothrix* complex species of clinical isolates could readily be recognized by ITS phylogeny.

The aims of this study were to (1) collect a wide range representative strains of pathogens causing sporotrichosis in China, and (2) characterize these strains and confirm their identity through analyses of the ITS sequences and β-tubulin of these strains with relevant taxa.

2 Materials and methods

2.1 Fungal isolates

Sixty-four clinical isolates identified as *S. schenckii sensu lato* were included in this study (Table 1). All these isolates were collected from the Dermatology Departments of three hospitals in China, mostly from farmers, housewives, or children. The breakdown is as follows: 26 from the First Hospital of Jilin University (Northeast China), 13 from the First Hospital of Beijing University (North China), and 25 from the Southwest Hospital of the Third Military Medical University (Southwest China). These isolates were subcultured on 2% potato-dextrose agar plates at 25 °C for 14 d. Isolates were then stored in sterilized water at 4 °C and on potato dextrose agar (PDA) slant at room temperature.

2.2 DNA extraction, amplification, and sequencing

Sporothrix spp. strains were inoculated on 2% PDA plates and incubated at 25 °C for 10 d. About 200 mg of fresh filamentous mycelia were scraped off for DNA extraction. The total genomic DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Porebski *et al.*, 1997). The quality and quantity of genomic DNA were detected by using 1% agarose gel electrophoresis imaging. Products were stored at −20 °C. The *BT2a*/*BT2b* primers were used to amplify the partial β-tubulin gene (Glass and Donaldson, 1995). The ITS region was amplified using the primer pair *ITS1*/*ITS4* (White *et al*., 1990). The polymerase chain reaction (PCR) mixture consisted of 2.5 μl of 10 mmol/L PCR buffer, 2 μl of 2.5 mmol/L dNTP mix, 1 μl of each primer (10 μmol/L), 1 μl of DNA template, 0.2 μl Taq DNA polymerase, and 17.3 μl double-distilled water. Amplification was performed through the following steps: initial denaturation at 95 °C for 5 min, followed by 35 cycling consisting of denaturing at 95 °C for 30 s, annealing at 52 °C (*Bt2*) or 54 °C (ITS) for 30 s, and extension at 72 °C for 1 min, followed by 10 min at 72 °C for extension. The PCR products were quantified by 1% agarose gel electrophoresis, and purified products were sequenced at the Beijing Genomics Institute (BGI; China) with the *ITS1*/*ITS4* and *BT2a*/*BT2b* primers mentioned above.

2.3 Sequence alignment and phylogenetic analysis

Sequences generated from the forward and reverse primers were assembled using a Contig Express component to obtain consensus sequence. The sequence identified for all our isolates was verified by a BLAST search (http://blast.ncbi.nlm.nih.gov). Several ITS regions and β-tubulin sequences published in GenBank were retrieved as reference sequences, together with sequences of new isolates (Table 1).

Alignments of ITS and combined ITS and β-tubulin were made using an MAFFT program (http://www.ebi.ac.uk/Tools/msa/mafft/) (Katoh and Toh, 2010), and followed by manual adjustments in BioEdit V7.0.5 (Hall, 1999). Both alignments were used for phylogenetic analyses.

The maximum parsimony (MP) analysis was performed using PAUP V.4.0b10 (Swofford, 2003). Ambiguously aligned regions were excluded from all analysis. Trees were inferred using the heuristic search by the 1000 random sequence addition and the tree-bisection-reconnection (TBR) branch-swapping algorithm. Branches of zero length were collapsed and all minimal-length trees were saved. Gaps were treated as missing data. Clade stability was assessed in a bootstrap test with 1000 replications. Tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI)

Table 1 Fungal species, strain numbers, origins, GenBank accession numbers, and references used in this study

Fungal species	Strain No.	Origin	GenBank No.		Reference
			ITS	β-tubulin	
S. globosa	LC2401	Clinical, Jilin, China	JX997723	KC407844	This study
S. globosa	LC2402	Clinical, Jilin, China	JX997718	KC407845	This study
S. globosa	LC2403	Clinical, Jilin, China	JX997741	KC407846	This study
S. globosa	LC2404	Clinical, Jilin, China	JX997737	KC407847	This study
S. globosa	LC2405	Clinical, Jilin, China	JX997702	KC407848	This study
S. globosa	LC2406	Clinical, Jilin, China	JX997734	KC407911	This study
S. globosa	LC2407	Clinical, Jilin, China	JX997738	KC407849	This study
S. globosa	LC2408	Clinical, Jilin, China	JX997715	KC407850	This study
S. globosa	LC2409	Clinical, Jilin, China	JX997740	KC407851	This study
S. globosa	LC2410	Clinical, Jilin, China	JX997711	KC407852	This study
S. globosa	LC2411	Clinical, Jilin, China	JX997731	KC407853	This study
S. globosa	LC2412	Clinical, Jilin, China	JX997733	KC407854	This study
S. globosa	LC2413	Clinical, Jilin, China	JX997713	KC407855	This study
S. globosa	LC2414	Clinical, Jilin, China	JX997712	KC407856	This study
S. globosa	LC2415	Clinical, Jilin, China	JX997730	KC407857	This study
S. globosa	LC2416	Clinical, Jilin, China	JX997709	KC407858	This study
S. globosa	LC2417	Clinical, Jilin, China	JX997710	KC407859	This study
S. globosa	LC2418	Clinical, Jilin, China	JX997724	KC407860	This study
S. globosa	LC2419	Clinical, Jilin, China	JX997719	KC407861	This study
S. globosa	LC2420	Clinical, Jilin, China	JX997720	KC407862	This study
S. globosa	LC2421	Clinical, Jilin, China	JX997728	KC407863	This study
S. globosa	LC2422	Clinical, Jilin, China	JX997698	KC407864	This study
S. globosa	LC2423	Clinical, Jilin, China	JX997739	KC407865	This study
S. globosa	LC2424	Clinical, Jilin, China	JX997729	KC407866	This study
S. globosa	LC2425	Clinical, Jilin, China	JX997735	KC407867	This study
S. globosa	LC2426	Clinical, Jilin, China	JX997700	KC407868	This study
S. globosa	LC2427	Clinical, Chongqing, China	JX997722	KC407869	This study
S. globosa	LC2428	Clinical, Chongqing, China	JX997692	KC407870	This study
S. globosa	LC2429	Clinical, Chongqing, China	JX997725	KC407871	This study
S. globosa	LC2430	Clinical, Chongqing, China	JX997717	KC407872	This study
S. globosa	LC2431	Clinical, Chongqing, China	JX997732	KC407873	This study
S. globosa	LC2432	Clinical, Chongqing, China	JX997704	KC407874	This study
S. globosa	LC2433	Clinical, Chongqing, China	JX997701	KC407875	This study
S. globosa	LC2434	Clinical, Chongqing, China	JX997705	KC407876	This study
S. globosa	LC2435	Clinical, Chongqing, China	JX997707	KC407877	This study
S. globosa	LC2436	Clinical, Chongqing, China	JX997742	KC407878	This study
S. globosa	LC2437	Clinical, Chongqing, China	JX997708	KC407879	This study
S. globosa	LC2438	Clinical, Chongqing, China	JX997721	KC407880	This study
S. globosa	LC2439	Clinical, Chongqing, China	JX997703	KC407881	This study
S. globosa	LC2440	Clinical, Chongqing, China	JX997695	KC407882	This study
S. globosa	LC2442	Clinical, Chongqing, China	JX997697	KC407884	This study
S. globosa	LC2443	Clinical, Chongqing, China	JX997693	KC407885	This study
S. globosa	LC2444	Clinical, Chongqing, China	JX997694	KC407886	This study
S. globosa	LC2445	Clinical, Chongqing, China	JX997726	KC407887	This study
S. globosa	LC2446	Clinical, Chongqing, China	JX997727	KC407888	This study

To be continued

Table 1

Fungal species	Strain No.	Origin	GenBank No.		Reference
			ITS	β-tubulin	
S. globosa	LC2447	Clinical, Chongqing, China	JX997716	KC407889	This study
S. globosa	LC2448	Clinical, Chongqing, China	JX997696	KC407890	This study
S. globosa	LC2449	Clinical, Chongqing, China	JX997699	KC407891	This study
S. globosa	LC2450	Clinical, Chongqing, China	JX997714	KC407892	This study
S. globosa	LC2451	Clinical, Chongqing, China	JX997736	KC407893	This study
S. globosa	LC2452	Clinical, Chongqing, China	JX997706	KC407894	This study
S. globosa	LC2453	Clinical, Beijing, China	KC407830	KC407895	This study
S. globosa	LC2454	Clinical, Beijing, China	KC407831	KC407896	This study
S. globosa	LC2455	Clinical, Beijing, China	KC407832	KC407897	This study
S. globosa	LC2456	Clinical, Beijing, China	KC407833	KC407898	This study
S. globosa	LC2459	Clinical, Beijing, China	KC407834	KC407900	This study
S. globosa	LC2460	Clinical, Beijing, China	KC407835	KC407901	This study
S. globosa	LC2462	Clinical, Beijing, China	KC407837	KC407903	This study
S. globosa	LC2463	Clinical, Beijing, China	KC407838	KC407904	This study
S. globosa	LC2465	Clinical, Beijing, China	KC407839	KC407906	This study
S. globosa	LC2466	Clinical, Beijing, China	KC407840	KC407907	This study
S. globosa	LC2467	Clinical, Beijing, China	KC407841	KC407908	This study
S. globosa	LC2468	Clinical, Beijing, China	KC407842	KC407909	This study
S. globosa	LC2469	Clinical, Beijing, China	KC407843	KC407910	This study
S. schenckii	CBS 117842 (CMW 7614)	Clinical, South Africa	AY280495	AY280477	Aghayeva *et al.* (2004)
S. schenckii	CMW 7615	Clinical, South Africa	AY280496	AY280478	Aghayeva *et al.* (2004)
S. schenckii	CMW 7612	Clinical, South Africa	AY280494	AY280476	Aghayeva *et al.* (2004)
S. schenckii	ATCC 26331 (CBS 359.36*)	Clinical, USA	FJ545232	AM116911	Marimon *et al.* (2006)
S. schenckii	CMW 5681	Clinical, South Africa	EF127886	EF139107	de Meyer *et al.* (2008)
S. brasiliensis	IPEC 17943	Clinical, Brazil	FN549902	AM116935	Madrid *et al.* (2010)
S. brasiliensis	IPEC 15572	Clinical, Brazil	FN549904	AM116955	Madrid *et al.* (2010)
S. globosa	FMR 8597	Clinical, Spain	FN549904	AM116964	Madrid *et al.* (2010)
S. globosa	CBS 120340* (FMR 8600)	Clinical, Spain	FN549905	AM116966	Madrid *et al.* (2010)
S. luriei	KMU 2787 (CBS 937.72*)	Clinical, South Africa	AB128012	AM747289	Kawasaki *et al.* (2003)
S. mexicana	CBS 120341*	Environmental, Mexico	FN549906	AM498344	Madrid *et al.* (2010)
S. pallida	CBS 150.87* (CMW 17168)	Environmental, Germany	EF127879	EF139109	de Meyer *et al.* (2008)
Ophiostoma nigrocarpum	CMW 650	*Abies* sp.	AY280489	AY280479	Aghayeva *et al.* (2004)

* Indicates the type strain. CBS: culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; CMW: culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa; ATCC: American Type Culture Collection, Virginia, USA; IPEC: Instituto de Pesquisa Clínica Evandro Chagas, Fiocruz, Brazil; FMR: Facultate de Medicina i Ciències de la Sault, Reus, Spain; KMU: Kanazawa Medical University, Ishikawa, Japan

were calculated for the generated parsimony trees. A Kishino-Hasegawa (KH) test was performed to determine whether the trees were significantly different. The trees were visualized in TreeView (Page, 1996). The model of evolution was estimated by using MrModeltest 2.3 (Nylander, 2004).

The newly generated sequences in this study were deposited in the GenBank, with their accession numbers listed in Table 1.

3 Results

Full length ITS regions and partial β-tubulin genes were amplified in this study. The amplified DNA fragments of the ITS regions and β-tubulin genes were approximatively 450 and 600 bp, respectively. The combined dataset of the ITS regions and β-tubulin genes contained 1052 characters, of which 742 characters were constant, 191 variable characters were parsimony-uninformative, and 119 were parsimony-informative. One of the most parsimonious trees with the shortest tree length (TL=465, CI=0.856, RI=0.835, RC=0.714, and HI=0.144) is shown in Fig. 1. In our combined phylogenetic tree, all the 64 clinical strains collected from different regions of China clustered in a well supported (99%) phylogenetic clade together with the ex-type strain of *S. globosa* (CBS 120340). This clade shows sufficient distance to the type of *S. schenckii* (CBS 359.36) (Fig. 1). The 64 isolates from China in the *S. globosa* clade were distributed among 18 different haplotypes.

4 Discussion

S. schenckii was attributed to a unique species as the pathogen of sporotrichosis since it was first isolated from a patient (Lopes-Bezerra *et al*., 2006). In past years, various molecular approaches have demonstrated that isolates of *S. schenckii* which were identified through morphology displayed diverse genetic characteristics and phylogenetic groupings (Lin *et al*., 2000; Mesa-Arango *et al*., 2002). Genotyping by analysis of restriction fragment length polymorphism (RFLP) of mitochondrial DNA (mtDNA) and ribosomal DNA (rDNA) demonstrated the existence of intraspecific genetic variability, and all of the four rDNA types showed different geographic distributions (Watanabe *et al*., 2004). With molecular phylogenetic analysis, *S. schenckii* was discovered to represent more than one species (de Beer *et al*., 2003). Marimon *et al.* (2007) revealed that *S. schenckii* was a complex of at least six phylogenetic species, comprising currently four clinical relevant species, i.e., *S. schenckii s. str.*, *S. brasiliensis*, *S. globosa* (Marimon *et al*., 2007), and *S. luriei* (Marimon *et al*., 2008b). Marimon *et al.* (2007) and Zhou *et al.* (2013) both revealed the existence of the correlation between geographical distribution and genotypes among species of *Sporothrix*. *S. brasiliensis* was confirmed to be restricted to Brazil, while *S. schenckii* was mainly prevalent in the Americas, Africa, and Asia (Marimon *et al*., 2007; Zhou *et al*., 2013).

S. globosa had been reported with widespread geographical distributions, such as in Chile, India, Italy, Japan, Spain, UK, and USA (Madrid *et al*., 2009). Previously, based on calmodulin (CAL) sequence analysis, five Chinese environmental isolates received as *S. schenckii* had been clustered into the clade of *S. globosa* (Marimon *et al*., 2007), together with six clinical isolates of *Sporothrix* using ITS region analysis (Zhou *et al*., 2013), and a total of 11 (100%) Chinese isolates were confirmed to be *S. globosa*. In this study, we sampled 64 clinical strains from a wider geographic regions of China, and our combined analysis of ITS and β-tubulin regions further confirmed that the causal agent of sporotrichosis is *S. globosa* in China. However, none of these isolates exhibited the same haplotype as the type of *S. globosa* CBS 120340, which is a clinical strain from Spain. We did not find a significant correlation between geographic origins of these strains and their genetic variations based on the haplotype data. More research is needed to indicate whether *S. schenckii* or other *Sporothrix* species exist in China.

Sporotrichosis is characterized by a wide range of cutaneous and extracutaneous clinical manifestations. Kong *et al.* (2006) revealed that different randomly amplified polymorphic DNA (RAPD) genotypes of strains led to different clinical forms. There have been reports about cutaneous dissemination and invasive sporotrichosis caused by *S. schenckii* and *S. brasiliensis* since Marimon *et al.* (2007) discovered the three new species of *Sporothrix* (Galhardo *et al*., 2010; Silva-Vergara *et al*., 2012). However, no case

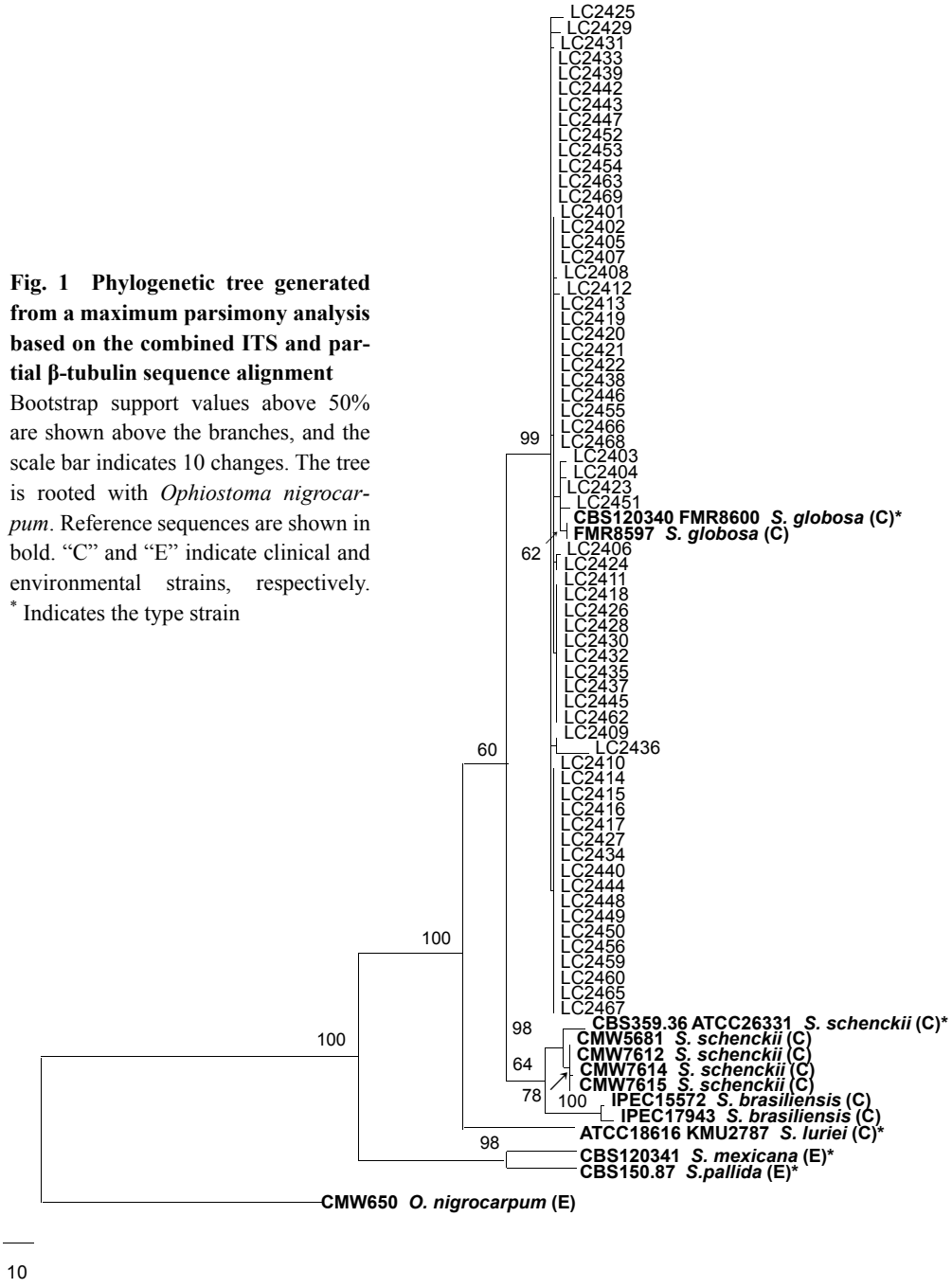

Fig. 1 Phylogenetic tree generated from a maximum parsimony analysis based on the combined ITS and partial β-tubulin sequence alignment

Bootstrap support values above 50% are shown above the branches, and the scale bar indicates 10 changes. The tree is rooted with *Ophiostoma nigrocarpum*. Reference sequences are shown in bold. "C" and "E" indicate clinical and environmental strains, respectively. * Indicates the type strain

of such clinical classification attributed to *S. globosa* has been reported. Except of the absence of sporotrichosis forms caused by *S. globosa* in some studies (Zhou *et al*., 2013), all of the clinical patterns in our study were characterized as fixed and lymphocutaneous, as well as five cases caused by *S. globosa* described in Brazil (de Oliveira *et al*., 2010; Rodrigues *et al*., 2012) and Chile (Cruz *et al*., 2012). We considered that this observation could be explained by the different virulence levels of members in *S. schenckii* complex. Although a study on the murine model has shown that the most virulent species was *S. brasiliensis*, followed by *S. schenckii* and *S. globosa* (Arrillaga-Moncrieff *et al*., 2009), further studies are needed to investigate the possible pathogenesis of different clinical types of sporotrichosis.

Some *in vitro* studies of *S. schenckii sensu lato* showed variable antifungal susceptibility among different isolates (Brandsberg and French, 1972; McGinnis *et al*., 2001), which indicates the existence of cryptic species with variations in antifungal susceptibility. Similar conclusions were presented by the studies of other fungal complexes such as the *Pseudallescheria boydii* (Gilgado *et al*., 2006) and the *Coccidioides immitis* complex (Koufopanou *et al*., 2001). In a recent *in vitro* antifungal susceptibility test, *S. brasiliensis* showed the best response to antifungals in general, followed by *S. schenckii*, *S. albicans*, *S. globosa*, and *S. mexicana* (Marimon *et al*., 2008a). Therefore, species identification of clinical isolates is essential to determine the effective treatment, and it is significant to study their epidemiology, geographic distribution, and the relevance among taxa, virulence, and clinical patterns.

5 Conclusions

The data in our study showed that the sporotrichosis cases in China were caused by *S. globosa* rather than *S. schenckii*, and it is demonstrated that ITS and β-tubulin are also efficient to distinguish species in *S. schenckii* species complex. With the increasing multilocus sequence data in this group, efficient molecular diagnosis protocols using specific primers should be developed to enable a rapid and accurate identification of clinical *Sporothrix* species.

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Compliance with ethics guidelines

Ting-ting LIU, Ke ZHANG, and Xun ZHOU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要：

本文题目：中国地区孢子丝菌临床分离株的分子鉴定

Molecular identification of *Sporothrix* clinical isolates in China

研究目的：明确中国地区孢子丝菌病的致病菌种。

创新要点：构建中国地区临床孢子丝菌系统进化树，用分子生物学方法对致病菌种进行重新鉴定。

研究方法：多基因测序和系统发育分析。

重要结论：球形孢子丝菌可能为中国地区孢子丝菌病的主要病原菌，申克孢子丝菌在中国地区是否存在尚需进一步验证。

关键词组：内含子转录间隔区；球形孢子丝菌；分类学；系统发育