Original Article

Studies in Phylogeny, Development of Rapid Identification Methods, Antifungal Susceptibility, and Growth Rates of Clinical Strains of *Sporothrix schenckii* Complex in Japan

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ABSTRACT

Sporotrichosis is a fungal infection caused by the *Sporothrix* species, which have distinct virulence profiles and geographic distributions. We performed a phylogenetic study in strains morphologically identified as *Sporothrix schenckii* from clinical specimens in Japan, which were preserved at the Medical Mycology Research Center, Chiba University. In addition, we examined the *in vitro* antifungal susceptibility and growth rate to evaluate their physiological features. Three hundred strains were examined using sequence analysis of the partial calmodulin gene, or polymerase chain reaction (PCR) method using newly designed species-specific primers; 291 strains were *Sporothrix globosa* and 9 strains were *S. schenckii* sensu stricto (in narrow sense, s. s.). *S. globosa* strains were further clustered into two subclades, and *S. schenckii* s. s. strains were divided into three subclades. In 38 strains of *S. globosa* for which antifungal profiles were determined, 4 strains (11%) showed high minimal inhibitory concentration (MIC) value for itraconazole. All tested strains of *S. schenckii* s. s. and *S. globosa* showed low sensitivity for amphotericin B. These antifungals are used for treatment of sporotrichosis when infection is severe. *S. schenckii* s. s. grew better than *S. globosa*; wherein *S. globosa* showed restricted growth at 35°C and did not grow at 37°C. Our molecular data showed that *S. globosa* is the main causal agent of sporotrichosis in Japan. It is important to determine the antifungal profiles of each case, in addition to accurate species-level identification, to strategize the therapy for sporotrichosis.

Key words : *Sporothrix schenckii* sensu stricto, *Sporothrix globosa*, sporotrichosis

Introduction

Sporotrichosis is a subacute or chronic mycosis that occurs in humans and animals worldwide. This infection is spread lymphatically or hematogenously depending on the immune status of the host, and most often, it presents as a localized lesion with subcutaneous nodules that eventually break through the skin^{1–3}.

This disease is caused by a dimorphic fungus, previously described as a single species – *Spor-*

othrix schenckii. The species is now recognized as *Sporothrix schenckii* complex, which comprises three phylogenetic sibling species, namely, *Sporothrix brasiliensis*, *Sporothrix globosa*, and *Sporothrix luriei*, in addition to *S. schenckii* sensu stricto (in a narrow sense, abbrev; s. s.), based on phylogenetic studies using DNA sequencing of specific regions of rDNA internal transcribed spacers (ITS) and calmodulin, β -tubulin, and translation elongation factor 1 α genes^{4–6}. In particular, the sequence of calmodulin gene has been widely used for taxonomy of *Sporothrix*

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Received : 5, February 2016 Accepted: 25, March 2016

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species⁵⁾.

These species have distinct virulence profiles and geographic distributions. *S. schenckii* s. s. and *S. brasiliensis* are more virulent in mice than *S. globosa*⁷⁻⁹⁾. *S. brasiliensis* is restricted geographically to Brazil, and *S. luriei* has only been reported to be isolated from a patient in South Africa. Conversely, *S. globosa* and *S. schenckii* s. s. show a global distribution, and they show differences in global frequency as causal agents of the disease^{4, 6, 10)}. *Sporothrix pallida* complex, which includes *Sporothrix chilensis*, *Sporothrix dimorphospora*, *Sporothrix humicola*, *Sporothrix mexicana*, *Sporothrix nivea*, and *Sporothrix stylites*, in addition to *S. pallida*, occupy a distant phylogenetic position from *S. schenckii* complex and are very rarely identified from clinical origins^{5, 10)}.

An effective therapy for localized sporotrichosis is a saturated solution of potassium iodide¹¹⁾. Other drugs commonly used are itraconazole (ITCZ) for the treatment of lymphocutaneous infections¹²⁻¹⁴⁾ and amphotericin B (AMHB) for severe infections or when ITCZ therapy fails¹⁵⁾.

Genotyping of *S. schenckii* complex in Japan has been performed based on restriction fragment length polymorphism of mitochondrial DNA^{16, 17)}. Recently, the MAT1-1: 1-2 ratio of *S. globosa* isolates in Japan was estimated to be 1: 3¹⁸⁾.

The aim of this study was to reinvestigate the phylogenetic position and the physiological features of the *S. schenckii* complex from clinical specimens in Japan in order to strategize the treatment for sporotrichosis. We developed polymerase chain reaction (PCR) methods using newly designed primer sets to rapidly discriminate *S. schenckii* s. s. and *S. globosa* at the species level, and identified 300 Japanese clinical strains of *S. schenckii* complex based on sequence analysis of the partial calmodulin gene or using our PCR methods. We also estimated the *in vitro* susceptibility to antifungal drugs and growth rates of 38 strains of *S. globosa*, which is more than in previous reports^{4, 19)}.

Material and methods

Fungal isolates and strains used

In this study, 300 clinical strains isolated from Japanese patients with sporotrichosis, previously identified as *S. schenckii* using traditional morphological analysis methods and preserved at the Medical Mycology Research Center, Chiba Uni-

versity, were re-examined using molecular methods (Table 1).

Identification based on sequence of calmodulin gene

Genomic DNA was extracted and purified from *Sporothrix* spp. mycelial phase cultivated on Potato Dextrose Agar (PDA-Difco™ Becton, Dickinson and Company/Sparks, MD, USA) slant using benzyl chloride method²⁰⁾.

Partial sequencing of the nuclear calmodulin gene was performed with primers CL1 (5-GA (GA) T (AT) CAAGGAGGCCTTCTC-3) and CL2A (5-TTTTTCAT CATGAGTTGGAC-3), as described by Marimon *et al.* (2007)⁴⁾. Samples were amplified using the following cycling parameters: one initial cycle of 10 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C, and a single extension cycle at 72°C for 10 min.

The PCR products were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM® 3130ABI Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. DNA sequences were edited using ATGC Ver. 4 sequence assembly software (Genetyx Co., Tokyo, Japan). The alignments of the sequences and phylogenetic trees based on neighbor-joining (NJ) analysis²¹⁾ were performed using Clustal X software²²⁾. The distances between sequences were calculated using Kimura's two-parameter model²³⁾. A bootstrap was conducted with 1,000 replications²⁴⁾.

Primer design

Four sets of primers were designed to distinguish *S. schenckii* s. s., *S. globosa*, *S. globosa* subgroup I and subgroup II by targeting the partial calmodulin gene region²⁵⁾. The particularly highly conserved site at the 3'-terminus in the target region was used to design species-specific primers. The following primers were designed for specific amplification of each species: *S. schenckii* s. s., SsF1 (5'-GCTAACCACTTTTTTTGTTC-3') and SsR1 (5'-TCCAGATCACCGTGTCTA-3'); *S. globosa*, SgF1 (5'-GCTAACCTCTTTCTTTGG-3') and SgR1 (5'-TGCCGCGCAACGGGAACCGAG-3'); *S. globosa* subgroup I, Sg1F1 (5'-CCGGTCGCCG CCATCGTACT-3') and SgR2 (5'-TACCCGTGCTG CGAATTGCC-3'); and *S. globosa* subgroup II, Sg2F1 (5'-GCACCCGGTCGCCGCCATTT-3') and SgR2. The degree of sequence identity between the designed primers and the calmodulin gene was analyzed using BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Table 1. Fungal isolates used in this study

Species	No. of strains	GenBank accession No. of calmodulin gene	Source	PCR detection				DNA template No.
				SsF1/ SsR1	SgF1/ SgR1	Sg1F1/ SgR2	Sg2F1/ SgR2	
<i>Sporothrix schenckii</i>	CBS 130114	KP101415	Human, Japan					
(AFLP schen A)	IFM 5890	LC115036	Human, Japan	+	-	-	-	
	IFM 50961	LC115037	Human, Japan	+	-	-	-	
<i>S. schenckii</i> (AFLP schen B)	CBS 117842	KP101387	Human, S-Africa					
<i>S. schenckii</i> (AFLP schen C)	CBS 130114	KP101392	Human, Peru					
	IFM 46302	LC115038	Human, Japan	+	-	-	-	2
<i>S. schenckii</i> (AFLP schen D)	CBS 938.72	KP101394	Human, France					
<i>S. schenckii</i> (AFLP schen E)	CBS 359.36 ^T	KP101420	NK, US	+	-	-	-	1
	IFM 41598	LC115039	Human, Japan	+	-	-	-	
	IFM 46307	LC115040	Human, Japan	+	-	-	-	
	IFM 46350	LC115041	Human, Japan	+	-	-	-	
	IFM 50962	LC115042	Human, Japan	+	-	-	-	
	IFM 50963	LC115043	Human, Japan	+	-	-	-	
	IFM 60241	LC115044	Human, Japan	+	-	-	-	
<i>S. globosa</i> (subgloup I)	CBS 120340 ^T	KP101459	Human, Japan	-	+	+	-	
	DMU1	KC190217	Human, China	-	+	+	-	
	IFM 5891	LC120835	Human, Japan	-	+	+	-	
	IFM 5897	LC120836	Human, Japan	-	+	+	-	
	IFM 5912	LC120837	Human, Japan	-	+	+	-	
	IFM 5930	LC120838	Human, Japan	-	+	+	-	
	IFM 5949	LC120839	Human, Japan	-	+	+	-	
	IFM 6046	LC120840	Human, Japan	-	+	+	-	
	IFM 46360	LC120663	Human, Japan	-	+	+	-	3
	IFM 46361	LC120664	Human, Japan	-	+	+	-	4
	IFM 50967	LC120843	Human, Japan					
	IFM 50968	LC120844	Human, Japan					
	IFM 50969	LC120845	Human, Japan					
	IFM 50972	LC120846	Human, Japan					
	IFM 50974	LC120847	Human, Japan					
	IFM 50975	LC120848	Human, Japan					
	IFM 50976	LC120849	Human, Japan					
	IFM 50977	LC120850	Human, Japan					
	IFM 50979	LC120851	Human, Japan					
	IFM 50980	LC120852	Human, Japan					
	IFM 54251	LC120853	Human, Japan					
	IFM 55519	LC120854	Human, Japan					
	IFM 55594	LC120855	Human, Japan					
	IFM 57547	LC120856	Human, Japan					
	IFM 58046	LC120857	Human, Japan					
	IFM 58181	LC120858	Human, Japan					
	IFM 58182	LC120859	Human, Japan					
	IFM 58187	LC120860	Human, Japan					
	IFM 58191	LC120861	Human, Japan					
	IFM 58499	LC120862	Human, Japan					

	IFM 59345	LC120863	Human, Japan					
	IFM 59446	LC120864	Human, Japan					
	IFM 60377	LC120865	Human, Japan					
	IFM 60781	LC120866	Human, Japan					
	IFM 60896	LC120867	Human, Japan					
	IFM 62673	LC120868	Human, Japan					
	IFM 62727	LC120869	Human, Japan					
	IFM 63340	LC120870	Human, Japan					
<i>S. globosa</i> (subgroup II)	205 strains		Human, Japan	-	+	+	-	
	DMU22	KC190217	Human, China					
	IFM 5986	LC120657	Human, Japan	-	+	-	+	6
	IFM 6015	LC120658	Human, Japan	-	+	-	+	5
	IFM 53929	LC122543	Human, Japan					
	IFM 55052	LC122544	Human, Japan					
	IFM 55520	LC122545	Human, Japan					
	IFM 58055	LC120655	Human, Japan	-	+	-	+	
	IFM 58089	LC120656	Human, Japan	-	+	-	+	
	IFM 61322	LC120659	Human, Japan	-	+	-	+	
	IFM 62244	LC120660	Human, Japan					
	IFM 63176	LC120661	Human, Japan					
	IFM 63204	LC120662	Human, Japan					
	39 strains		Human, Japan	-	+	-	+	
<i>S. brasiliensis</i>	CBS 120339 ^T	KP101421	Human, Brazil	-	-	-	-	8
<i>S. luriei</i>	CBS 937.72 ^T	AM747302	Human, S-Africa	-	-	-	-	7
<i>S. mexicana</i>	CBS 12034 ^T	AM398393	Soil, Mexico					
<i>S. pallida</i>	CBS 302.73	AM398396	Soil, S-Africa	-	-	-	-	9

T; ex type. S-Africa; South Africa.

PCR amplification

DNA solutions were diluted to 10 ng/μl with distilled water. PCR mixtures were prepared using each primer set, SsF1/ SsR1, SgF1/SgR1, Sg1F1/ SgR2, or Sg2F1/SgR2 primers, and SapphireAmp Fast PCR Master Mix (Takara Bio). Amplifications for SsF1/ SsR1 or SgF1/SgR1 were performed in a Thermal Cycler Dice (Takara Bio) with initial heat activation at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Amplifications for Sg1F1/SgR2 or Sg2F1/SgR2 were changed to initial heat activation at 96°C for 10 min followed by 30 cycles of denaturation at 96°C for 1 min, annealing at 5°C for 1 min, and extension at 72°C for 1 min. The entire reaction solution was applied to a 1.5% agarose gel and separated electrophoretically, and the gels were stained in ethidium bromide prior to visualization using a UV transilluminator.

Culture conditions for antifungal susceptibility tests

Many isolates of *S. globosa* did not grow at 37 or 35°C on brain-heart infusion (BHI) agar slants, which were generally considered suitable for analysis of antifungal profiles in yeast phase. Even after successful subculture, some isolates did not grow sufficiently for identification in antifungal susceptibility tests after culture at 35°C for 72 h, and extension of culture period was not appropriate for tests following the antifungal susceptibility method (antifungal profiles were quite different particularly for those in which the endpoint was defined as 50% growth inhibition). Therefore, we selected *S. globosa* strains growing on BHI agar slants at 35°C, and culture conditions were further analyzed with fixed culture period at 72 h: BHI agar slant at 35°C (subculture) with culture at 35°C for 72 h; PDA slants at 25°C (subculture) with culture at 35°C for 72 h, PDA slants at 25°C (subculture) with culture at 30°C for 72 h.

Fig. 1. Neighbor-joining tree based on partial calmodulin gene sequences for *Sporothrix schenckii* complex and related species. Bootstrap based on 1,000 samplings supporting the internal branches with a probability of 50% or higher are shown.

Antifungal susceptibility

Minimal inhibitory concentration (MIC) and minimal effective concentration (MEC) of antifungal agents against the isolated fungi were measured using the microdilution method, based on the Standardized Clinical and Laboratory Standards Institute M38-A2 Method (CLSI M38-A2) for filamentous fungi²⁶⁾ with a slight modification (temperature was adjusted to 30°C). Microtiter plates (Dry Plate; Eiken Chemicals, Tokyo, Japan) containing lyophilized antifungals that were serially diluted two-fold were used according to the manufacturer's instructions. Forty-seven

strains were subcultured on PDA slants for 7 days at 25°C. The inoculum suspension was adjusted to 2×10^4 cells/ml in RPMI 1640 broth, added to the microtiter wells at 0.1 ml, and then incubated at 30°C for 72 h. Visual examination of growth inhibition was performed.

The MIC endpoint for AMPH, ITCZ, and voriconazole (VRCZ) was defined as the lowest concentration that produced complete inhibition of growth, and for flucytosine (5-FC), fluconazole (FLCZ), and miconazole (MCZ) as the lowest concentration that produced 50% growth inhibition. The MEC for micafungin (MCFG) was de-

defined as the lowest concentration that reduces fungal growth compared to controls.

Growth rates

In order to study thermotolerance, 45 isolates were inoculated on PDA plates and incubated at 30, 35, and 37°C in the dark for 3 weeks. The petri dishes were inoculated with 10 µl of the conidial suspension, which was adjusted to 2×10^4 cells/ml for each isolate and then placed upside down. The colony diameters (in mm) were measured after 21 days of incubation.

Results

Sequence analysis

New sequences were deposited in the DNA Data Bank of Japan (DDBJ), and the accession numbers are shown in Table 1. Fig. 1 shows the phylogenetic tree of *S. schenckii* s.s., *S. globosa*, and related species based on the partial calmodulin gene sequences. *S. schenckii* s.s. strains and *S. globosa* strains were clustered together in each monophyletic clade, supported with high bootstrap values. Forty-seven of 56 clinical strains in Japan were clustered with *S. globosa*. Moreover, these isolates were divided into two highly supported subclades: *S. globosa* I and *S. globosa* II by Yu *et al.* (2013)²⁶⁾. *S. globosa* I consisted of 36 strains, while *S. globosa* II consisted of 11 strains. *S. schenckii* s.s. clade, including nine Japanese strains and reference strains, were divided into five subclades, AFLP schen A-E reported by Zhang *et al.* (2015)¹⁰⁾ and Japanese strains were clustered with AFLP schen A (2 strains), C (1 strain), and E (6 strains).

PCR using newly designed specific primers

Using the SsF1/ SsR1 primer set designed for *S. schenckii* s. s. or the SgF1/ SgR1 primer set designed for *S. globosa*, we subjected 251 strains to PCR-based assay, and amplification products of approximately 400 bp or 100 bp were detected for five strains of *S. schenckii* s. s. or 246 strains of *S. globosa*, respectively (Fig. 2). No PCR products were obtained for other species of *Sporothrix*. Using the Sg1F1/ SgR2 primer set designed for *S. globosa* I or the Sg2F1/ SgR2 primer set designed for *S. globosa* II, we subjected 246 strains to PCR-based assay, and amplification products of approximately 300 bp were detected for 205 strains of *S. globosa* I and 41 strains of *S. globosa* II. Sequences of all strains of *S. schenckii* s. s. were determined.

Fig. 2. PCR amplifications using species-specific primers SsF1/SsR1, SgF1/SgR1, Sg1F1/SgR2, and Sg2F1/SgR2. The numbers at the top of each lane (1 to 9) correspond to Table 1. M: 100-bp ladder.

Antifungal susceptibility

The outcomes from three tested conditions did not differ substantially and did not show any specific tendencies. Therefore, we chose the following standardized conditions for our antifungal susceptibility tests: PDA slants at 25°C (sub-culture) with culture at 30°C for 72 h.

The MIC or MEC of antifungal agents against the strains are listed in Table 2.

Growth rates

The growth rates of the strains are listed in Table 3. *S. schenckii* s. s. grew better than *S. globosa* especially at 35°C and 37°C. The colonies of *S. schenckii* strains when grown on PDA attained a diameter of 19-25 mm at 35°C in 21 days, while growth rate of *S. globosa* strains was restricted and classified into three patterns: no growth, slight growth, and a diameter of 8-16 mm.

Table 2. MICs or MEC ($\mu\text{g/ml}$) of species of *Sporothrix schenckii* complex in Japan

	MCFG	AMPH	5-FC	FLCZ	ITCZ	VRCZ	MCZ
<i>S. globosa</i>							
Subgroup I (29 strains)	0.12–1	1–4	0.12–32(> 128)	> 128(32)	0.5–4	> 16(8)	0.03–1
Subgroup II (9 strains)	0.12–0.5	2–4	0.5–64	> 128	0.25–2(8, > 16)	> 16	0.12–1
<i>S. schenckii</i> (9 strains)	0.12–0.25(> 16)	2	0.5–4	> 128	0.5–1	> 16(8)	0.03–0.5

(); exceptional concentrations. AMPH; amphotericin B, 5-FC; 5-flucytosine, FLCZ; fluconazole, ITCZ; itraconazole, VRCZ; voliconazole, MCZ; miconazole, MCFG; micafungin.

Table 3. Growth rate (mm) of species of *Sporothrix schenckii* complex in Japan

		30°C	35°C			37°C		
			Growth	Slight growth	No growth	Growth	Slight growth	No growth
<i>S. globosa</i>								
Subgroup I (29)	Range	22–42 (29)	8–16 (15)	(12)	(2)			(29)
	Mean	34.3	10.8					
Subgroup II (9)	Range	26–41 (9)	10 (2)	(4)	(3)			(9)
	Mean	32.9	10					
<i>S. schenckii</i> (7)	Range	34–48 (7)	18–25 (7)			7–16 (5)	(1)	(1)
	Mean	37.5	21.3			10.7		

(); No. of tested strains.

In addition, all tested *S. globosa* strains did not grow at 37°C.

Discussion

The *Sporothrix* species show differences in global frequency as causal agents of the disease. In East Asia, *S. globosa* is the main endemic species, while in America, Australia, and South Africa, *S. schenckii* s. s. is the main species, and *S. braziliensis* is the main species in Brazil¹⁰. Our phylogenetic analysis based on sequence analysis and PCR method showed that 291 of the 300 clinical strains in Japan were clustered with *S. globosa*, which were further subdivided into two subgroups. *S. globosa* subgroup I (241 strains) was significantly more frequent than subgroup II (50 strains). The endemicity of *S. globosa* subgroup I and II in Japan was similar to that in China²⁵, while *S. globosa* isolates from Brazil were homogenous¹⁰ and belonged to only subgroup I in our analysis. It may be related to areal differentiation of *S. globosa*.

Meanwhile, it is notable that 9 of the 300 Japanese clinical isolates were clustered with *S.*

schenckii s. s., while all isolates in China were entirely clustered with *S. globosa*^{25, 27}. Zhang *et al.* (2015)¹⁰ reported that *S. schenckii* s. s. were divided into five subclades, AFLP schen A to E, using phylogenetic analysis based on concatenated calmodulin and translation elongation factor -1 and -3 genes. We also observed grouping into these subclades in our study based on only the calmodulin gene, which is widely used for taxonomy of *Sporothrix* species⁵. Six of the 9 Japanese strains were clustered with AFLP schen E, including the type strain of *S. schenckii* s. s., while most strains in South and Central America were clustered with AFLP schen A. Genotypes of *S. schenckii* s. s. also seem to be related to geography.

There were no clear relationships between genotypes (*S. globosa* subgroup I, II or *S. schenckii* s. s.) and geography in Japan or clinical forms, since clinical course is also affected by immune status of the host. From a review of literature, we found that conditions for antifungal susceptibility tests generally vary substantially. For example, Marimon *et al.*¹¹, who evaluated *S. schenckii* species complex, estimated the antifungal profiles

for sibling species at 30°C for 72 h (subculture on PDA at 30°C). Trilles *et al.*²⁸⁾ and Gutierrez-Galhardo *et al.*²⁹⁾ compared the differences in antifungal profiles between the mycelial phase and yeast phase, similar to our previous experimental design; however, these reports only identified isolates based on morphological and microscopic appearance, and there was no information regarding how many *S. globosa* isolates were included. Moreover, Trilles *et al.*²⁸⁾ incubated yeast phase at 35°C, but evaluated the results after 6 days of incubation. Gutierrez-Galhardo *et al.*²⁹⁾ tested the conditions at 35°C for 72 h for the yeast phase; however, they used the Antifungal Susceptibility Testing of the European Committee for Antimicrobial Susceptibility Testing (AFST-EUCAST), in which the inoculum and nutrients are 10-fold higher than those in the CLSI methods, and incubated the cultures with a shaker to improve growth only for the yeast phase. Rodrigues *et al.*¹⁹⁾ estimated growth using the CLSI method with incubation at 35°C for 72 h; however, the majority of isolates were *S. brasiliensis* (22 isolates) and *S. schenckii* s. s. (39 isolates), and only four isolates of *S. globosa* were included in their results. *S. globosa* has been less analyzed than *S. schenckii* s.s. or *S. brasiliensis* until recently because it is not the main species in America or Europe, and *S. schenckii* s.s. and *S. brasiliensis* grow well at 35°C⁴⁾. The CLSI method is the most popular for antifungal susceptibility tests. However, this test is generally only recommended for filamentous fungi, not for *S. schenckii* complex. Considering the thermotolerance of *S. globosa*, it is suggested that incubation at 30°C for 72 h is more suitable rather than incubation at 35°C as the culture conditions for antifungal susceptibility testing with the *S. schenckii* complex.

Compared to *S. schenckii* s. s. and *S. brasiliensis*, reports about the *in vitro* antifungal profiles for *S. globosa* are relatively scarce and are based on a small number of tested strains. In this study, we determined antifungal profiles for 38 strains of *S. globosa* in Japan. Our results showed that 4 strains (11%) of *S. globosa* showed high MIC value (4-> 16 µg/ml) for ITCZ, and all tested strains of *S. schenckii* s. s. and *S. globosa* showed low sensitivity for AMPH (1-4 µg/ml); these antifungals are commonly used for treatment of sporotrichosis when infection is severe¹²⁻¹⁵⁾. Although breakpoints have not been established for *S. schenckii* complex species, CLSI M38-A2 suggests

that, for analytical purposes, filamentous fungi with an MIC > 4.0 µg/ml for ITCZ may be considered resistant³⁰⁾. In this study, all tested *S. schenckii* s. s. strains showed low MIC for ITCZ, although Stopigila *et al.*³⁰⁾ have reported 4 of 61 *S. schenckii* s. s. isolates in Brazil that showed high MIC for ITCZ. They also reported that 1 of 4 *S. globosa* isolates showed high MIC for ITCZ and concluded that the result was in agreement with previous descriptions of the use of the drug in clinical settings, as 92% cure was achieved with the use of this antifungal^{31, 32)}.

Further, all tested strains in this study showed high MIC for FLCZ and VRCZ, which coincided with the results reported in previous studies^{28, 29, 33, 34)}. FLCZ is considered as the second-line treatment for sporotrichosis³⁵⁾. In spite of the poor activity that this drug has shown in this and other studies, its clinical efficacy has been estimated to be 71% for cases of lymphocutaneous infection³⁶⁾.

Although *in vitro* results do not always correlate with *in vivo* outcomes, it is notable that strains showing high MIC for ITCZ also show high MIC or resistance for AMPH, FLCZ, and VRCZ. Therefore, it is important to determine the antifungal profiles of each case, in addition to accurate species-level identification.

Some *S. globosa* isolates grew as well as *S. schenckii* s. s. at 30°C, but they showed very restricted growth at 35°C and could not grow at 37°C, while all tested *S. schenckii* s. s. isolates grew with colonies of 20 mm in diameter at 35°C. Some studies have shown that *S. globosa* do not grow at 37°C, while some studies have shown that they do grow^{4, 25)} — Yu *et al.*²⁵⁾ suggested that it depends on the inoculum preparation.

Kwon-Chung³⁷⁾ discovered that strains causing fixed cutaneous sporotrichosis grow best at 35°C, while those causing lymphocutaneous sporotrichosis grow at both 37°C and 35°C. Further, Yu *et al.*²⁵⁾ reported that isolates able to grow at 37°C could be obtained from fixed cutaneous, lymphocutaneous, or disseminated sporotrichosis. Mehta *et al.*³⁸⁾ observed that isolates from both fixed and lymphocutaneous types grew well at 37°C, suggesting that isolates that grow at 37°C were disseminated more easily than isolates that cannot grow at 37°C. In general, thermotherapy is very effective for fixed cutaneous sporotrichosis in Japan. Our result may show a reason for this, i. e., most sporotrichosis in Japan are caused by *S.*

globosa, which did not grow at 37°C. Virulence has been tested in animal inoculations using mice as model animal. Arrillaga *et al.*⁸⁾ reported that virulence of *S. schenckii* s. s. was higher than that of *S. globosa*. Our result suggests that the difference in thermotolerance can be a reason for the difference in the virulence level between the two species.

In Japan, an epidemic of sporotrichosis has not occurred in decades, but sporadic cases have been reported in the northern district where sporotrichosis is considered to occur very occasionally because of severe coldness or snow in winter, which is supposed to be caused by global warming. In addition, cats are very popular companion animals in Japan, and sporotrichosis caused by *S. brasiliensis*, which is significantly associated with cat-transmission¹⁰⁾, may occur in Japan because of the development of international distribution, like in the case of tinea corporis by *Arthroderma benhamiae* from rabbit. Additionally, Sporotrichosis can become more frequent or severe because of the increasing population of aged people or immunocompromised patients. Therefore, sporotrichosis is important as a re-emerging or imported infectious disease.

Worldwide, sporotrichosis is an epidemic that needs attention in accordance with the geography of the species. The antifungal susceptibility test showed that the test conditions and assessment criteria for *Sporothrix* have not been established yet, and our result suggests that clinical forms of sporotrichosis may be different between species because of their difference in thermotolerance. Future studies are required to analyze clinical cases based on molecular phylogeny. In this study, we reinvestigated the phylogenetic position of *S. schenckii* complex of Japanese clinical strains, as well as their physiological features. In the treatment of infectious disease, detecting the etiological agent to the species level is essential because accurate diagnosis is very important not only for clinical treatment but also for the epidemiology. We believe that PCR using our newly designed primer sets for identification between *S. globosa* and *S. schenckii* s. s. is very useful as it is simple and rapid compared to traditional species-level identification based on sequence analysis, which is time consuming and expensive.

Acknowledgements

This work was supported in part by the National Bioresource Project-Pathogenic microbes in Japan (<http://www.nbrp.jp/>).

Conflict of Interest

All authors declare no conflict of interest.

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