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Global ITS diversity in the *Sporothrix schenckii* complex

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Abstract Molecular phylogeny has revealed that sporotrichosis is caused by several *Sporothrix* species which differ in clinical behavior. The complex is embedded within *Ophiostoma*, a genus mainly comprising fungi that live in association with bark beetles, but differs by a high virulence towards humans and other mammals. The different ecology is corroborated by phylogenetic separation. The aim of the present study was to determine the validity of the rDNA internal transcribed spacer (ITS) region as a marker for diagnostics of species in the clinical group, using beta-tubulin sequences to calibrate species delimitations. The topology of the two gene trees was concordant, and all clinically relevant *Sporothrix* species could easily be recognized by means of the ITS region. An increased geographic sampling did not affected delimitation success in the clinical clade of the *S. schenckii* complex.

Keywords *Sporothrix schenckii* · Sporotrichosis · Taxonomy · Phylogeny · ITS · Barcoding

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Introduction

Sporothrix stands for simply structured, single-celled conidia on clusters of denticles. This morphology is expressed in numerous species of the order Ophiostomatales. Main teleomorph genus is *Ophiostoma*, a large group of pathogens of woody plants characteristically associated with bark-beetles (Zhou et al. 2006; Zipfel et al. 2006; Roets et al. 2006). The slimy ascospores and (syn)anamorphic conidia classified in *Sporothrix*, *Hyalorhinocladiella* and *Pesotum* each have particular roles in this specific habitat (Zipfel et al. 2006). Among the few exceptions with an entirely different ecology within the Ophiostomatales is *Sporothrix schenckii*, a widespread pathogen of humans and other mammals (Guarro et al. 1999).

Sporothrix schenckii s.l. is responsible for sporotrichosis, a chronic, granulomatous, cutaneous or subcutaneous infection particularly occurring in humans and cats. The most common route of infection is via traumatic implantation into the skin of otherwise healthy individuals. In immunocompromised patients systemic and disseminated infections are observed, affecting multiple organs (Callens et al. 2006; Silva-Vergara et al. 2012). *Sporothrix* infections may take

epidemic proportions (Dixon et al. 1991; Marimon et al. 2006; Verma et al. 2012). The source of human infection is environmental, mostly from soil and plant debris (Dixon et al. 1991; Mesa-Arango et al. 2002) and may also be transmitted from scratches by asymptomatic or infected cats (Schubach et al. 2004; Rosa et al. 2005; Barros et al. 2010).

The generic type species, *S. schenckii* is characterized by tear-shaped conidia on small, clustered denticles (de Hoog et al. 2000). The fungus is thermally dimorphic, i.e., at 37 °C a yeast-like phase is produced under appropriate conditions (Howard 1961; de Hoog 1974; Travassos and Lloyd 1980). Molecular phylogenetic analyses have shown that several species exist within the *S. schenckii* species complex (de Beer et al. 2003; Marimon et al. 2006, 2007; Madrid et al. 2010; Criseo and Romeo 2010). Multilocus sequence data proved to be supported by small phenotypic characters, which led to the description of the following novel clinically relevant species: *S. brasiliensis*, *S. globosa, S. mexicana* in addition to *S. schenckii s. str.* and *S. luriei* (Marimon et al. 2007, 2008). Recently described environmental species include *S. stylites, S. lignivora, S. humicola* (de Meyer et al. 2008), *S. variecibatus* (Roets et al. 2008), *S. brunneoviolacea* and *S. dimorphospora* (Madrid et al. 2010). The species differ significantly in virulence and predilection (Marimon et al. 2007; Arrillaga-Moncrieff et al. 2009; Fernández-Silva et al. 2012; Fernandes et al. 2013), and geographic distributions of some of the species are limited (Rodrigues et al. 2012).

The position of the *S. schenckii* complex amidst species with a rather consistent, entirely different ecology is puzzling. Some scattered case reports have been published in *Ophiostoma*, such as cutaneous and nail infections by *O. stenoceras* (Mariat 1971; Summerbell 1993) and a systemic infection in a leukemic patient by *O. piceae* (Bommer et al. 2009), but a human-pathogenic potential as in *Sporothrix* is absent from *Ophiostoma*.

Clinical *Sporothrix* species presently are classified with the aid of partial calmodulin sequences (Marimon et al. 2007). *Sporothrix schenckii* in a restricted sense still contains significant diversity (Marimon et al. 2006, 2007) compared to e.g. *S. brasiliensis* (Marimon et al. 2007; Rodrigues et al. 2012). This may reduce the barcoding gap, which is the ratio of intra- and interspecific variabilities, and hamper the development of specific barcode-identifiers (Heinrichs et al. 2012). The aim of the present study was to determine the validity of the internal transcribed spacer (ITS) region as a marker for species recognition in the *S. schenckii* complex. We evaluated an epidemiologically diverse strain panel of clinical and environmental isolates classified in 30 taxa with a global distribution and comprising both anamorph and teleomorph taxa.

Materials and methods

Fungal isolates

A total of 124 isolates identified as *Sporothrix* and *Ophiostoma* species (68 clinical, 56 environmental) by morphology and/or partial β-tubulin (*BT2*) sequence were included in the study (Table 1). Strains were provided by reference collections of ATCC, CBS, CMW, CNM-CM, IPEC and KMU. Ex-type strains were included for all species.

DNA extraction

Isolates were grown for 10 day on potato dextrose agar (PDA). DNA was extracted following the Quick CTAB extraction: Two mL screw-capped tubes were filled with 490 μL CTAB-buffer 2× and 6–10 acid-washed glass beads, 1–10 mm^3 fungal material was added, and 10 μL Proteinase K; the material was mixed thoroughly on a MoBio vortex for 10 min. After incubation for 60 min at 60 °C. 500 μL chloroform: isoamylalcohol (24:1) was added and shaken for 2 min. Tubes were centrifuged at 14,000 r.p.m. for 10 min. The upper layer was collected in a fresh tube. To ~400 μL DNA sample 2/3 vol (~270 μL) of ice cold iso-propanol was added and spun again at 14,000 r.p.m. for 10 min and samples were dissolved in 1 mL ice-cold 70 % ethanol. Tubes were spun again at 14,000 r.p.m. for 2 min, samples were air-dried and resuspended in 50 μL TE-buffer. Samples were stored −20 °C until use. Quality of DNA was verified by running 2–3 μL on a 0.8 % agarose gel.

PCR amplification

Sporothrix/Ophiostoma ITS regions were directly amplified from genomic DNA with primers ITS5 5′-GGA AGT AAA AGT CGT AAC AAG G-3′ and ITS4 5′-TCC TCC GCT TAT TGA TAT GC-3′ as described by Madrid et al. (2010). *BT2* region was amplified using the primers Bt2-F 5′-GG (CT) AACCA (AG) AT (ATC) GGTGC (CT) GC (CT)-3′ and Bt2-R 5′-ACC CTC (AG) GTG TAG TGA CCC TTG GC-3′ according to Marimon et al. (2006). The reaction mixture (25 μL final vol) contained PCR buffer (10×) 2.5 μL, water 15 μL, dNTP mix (1 mM) 2.5 μL, 1 μL of each primer (10 pmol), *Taq* polymerase (0.5 U) 1 μL, BSA (Bovine serum albumin) 1 μL, and DNA 1 μL [100 ng/μL]. PCR reactions were performed in a Hybaid Touchdown PCR machine (Hybaid, Middlesex, U.K.). PCR conditions were: one cycle of 5 min at 95 °C, followed by 35 cycles of 35 s at 95 °C, 30 s at 52 °C (ITS) or 60 °C (*BT2*) and 1 min at 72 °C, followed by one cycle of 6 min at 72 °C. PCR products were visualized by electrophoresis on a 1 % (w/v) agarose gel. PCR products were purified with the High Pure

Table 1 *Ophiostoma/Sporothrix* isolates included in the study

Species	CBS number, cross reference	Source	Origin	Genbank		ITS Reference	BT2 Reference
				ITS	BT2		
Ophiostoma abietinum	CBS 125.89 (C696)	*Abies vejari*	Mexico	AF484453	EU977484	de Beer et al. (2003)	Massoumi et al. (2009)
O. africanum	CBS 116566 (CMW 1104)	*Protea caffra*	South Africa	DQ316200	DQ316162	Roets et al. (2006)	Roets et al. (2006)
	CBS 116374 (CMW 1822)	*Protea dracomontana*	South Africa	DQ316197	DQ316159	Roets et al. (2006)	Roets et al. (2006)
O. aurorae	CMW 19362^{T}	*Pinus elliottii*	South Africa	DQ396796	DQ396800	Zhou et al. (2006)	Zhou et al. (2006)
O. bragantinum	CBS 474.91^{T}	Soil	Brazil	FN546965	FN547387	Madrid et al. (2010)	Madrid et al. (2010)
	CBS 430.92	Soil	Brazil	FN546964	FN547386	Madrid et al. (2010)	Madrid et al. (2010)
O. dentifundum	CMW13016	Quercus wood	Hungary	AY495434	AY495445	Aghayeva et al. (2005)	Aghayeva et al. (2005)
	CMW13017	Quercus wood	Poland	AY495435	AY495446	Aghayeva et al. (2005)	Aghayeva et al. (2005)
O. fusiforme	CBS 112909 (CMW 8281)	*Castanea sativa*	Azerbaijan	AY280482	AY280462	Aghayeva et al. (2004)	Aghayeva et al. (2004)
	CBS 112910 (CMW 8285)	*Castanea sativa*	Azerbaijan	AY280483	AY280463	Aghayeva et al. (2004)	Aghayeva et al. (2004)
O. lunatum	CBS 112928 (CMW 10564)	*Larix decidua*	Austria	AY280486	AY280467	Aghayeva et al. (2004)	Aghayeva et al. (2004)
	CBS 112927^{T} (CMW 10563)	*Carpinus betulus*	Austria	AY280485	AY280466	Aghayeva et al. (2004)	Aghayeva et al. (2004)
O. narcissi	CBS 138.50	Narcissus	Netherlands	AY194510	–	Jacobs et al. (2003)	–
O. nigrocarpum	CMW 651	*Pseudotsuga menziesii*	USA	AY280490	AY280480	Aghayeva et al. (2004)	Aghayeva et al. (2004)
	CMW 650^{T}	*Abies* sp.	USA	AY280489	AY280479	Aghayeva et al. (2004)	Aghayeva et al. (2004)
O. palmiculminatum	CBS 119590 (CMW20677)	Protea repens	South Africa	DQ316191	DQ821543	Roets et al. (2006)	Roets et al. (2008)
O. phasma	CMW 20686	*Protea laurifolia*	South Africa	DQ316223	DQ316185	Roets et al. (2006)	Roets et al. (2006)
	CBS 119721^{T} (CMW 20676)	*Protea laurifolia*	South Africa	DQ316219	DQ821541	Roets et al. (2006)	Roets et al. (2008)
O. piceae	CBS 10821^{T}	–	Germany	AF198226	–	Harrington et al. (2001)	–
O. protearum	CBS 116567 (CMW 1103)	*Protea caffra*	South Africa	DQ316203	DQ316165	Roets et al. (2006)	Roets et al. (2006)
	CBS 116654 (CMW 1107)	*Protea caffra*	South Africa	DQ316201	DQ316163	Roets et al. (2006)	Roets et al. (2006)
O. splendens	CBS 116569 (CMW 872)	Protea repens	NK	DQ316215	DQ836011	Roets et al. (2006)	Roets et al. (2008)
O. stenoceras	CMW 11193 (C966)	Wood	New Zealand	AY280493	AY280475	Aghayeva et al. (2005)	Aghayeva et al. (2004)
	CBS 237.32 (CMW 3202)	Pine pulp	Norway	AF484462	DQ296074	de Beer et al. (2003)	Zipfel et al. (2006)
	CMW 3998	Soil	Kenya	AF484463	–	de Beer et al. (2003)	–
Sporothrix brasiliensis	CBS 130108 (FMR8324, IPEC17943)	Clinical strain, human sporotrichosis	Brazil	KC113211	AM116935	This study	Marimon et al. (2006)
	CBS 130106 (FMR8304, IPEC15572)	Clinical strain, human sporotrichosis	Brazil	KC113212	AM116955	This study	Marimon et al. (2006)
	CBS 130109 (FMR8334, IPEC22582)	Clinical strain, human sporotrichosis	Brazil	KC113213	AM116956	This study	Marimon et al. (2006)
	CBS 130107 (FMR8318, IPEC17521)	Clinical strain, human sporotrichosis	Brazil	KC113214	AM116952	This study	Marimon et al. (2006)
	CBS 120339^{T} (IPEC 16490)	Clinical strain, human sporotrichosis	Brazil	–	AM116946	–	Marimon et al. (2006)
	IPEC 17943	Clinical strain, human sporotrichosis	Brazil	FN549902	AM116935	Madrid et al. (2010)	Marimon et al. (2006)
	IPEC 15572	Clinical strain, human sporotrichosis	Brazil	FN549903	AM116955	Madrid et al. (2010)	Marimon et al. (2006)
	CNMCM 3477	Deep sporotrichosis	Brazil	EU126945	–	Galhardo et al. (2008)	–
	CNMCM 3450	Clinical strain, human sporotrichosis	Brazil	EU126940	–	Galhardo et al. (2008)	–

Table 1 (continued)

Species	CBS number, cross reference	Source	Origin	Genbank		ITS Reference	BT2 Reference
				ITS	BT2		
	CNMCM 3453	Clinical strain from patient with mucocutaneous sporotrichosis	Brazil	EU126941	–	Galhardo et al. (2008)	–
	CNMCM 3457	Clinical strain from patient with sporotrichosis	Brazil	EU126942	–	Galhardo et al. (2008)	–
	CNMCM 3462	Patient with erythema nodosum	Brazil	EU126943	–	Galhardo et al. (2008)	–
	CNMCM 3469	Clinical strain, human sporotrichosis	Brazil	EU126944	–	Galhardo et al. (2008)	–
	ATCC MYA4823	Feline sporotrichosis	Brazil	JQ070115	–	–	–
S. brunneoviolacea	CBS 124561^{T} (FMR 9338)	Soil	Spain	FN546959	FN547385	Madrid et al. (2010)	Madrid et al. (2010)
	CBS 101570	Endophyte in *Vitis vinifera*	USA	KC113235	–	This study	–
	ATT 163	*Atta texana* nest	USA	HQ607869	–	Rodrigues et al. (2011)	–
S. dimorphospora	CBS553.74^{T}	Soil	Canada	AY495428	AY495439	Aghayeva et al. (2005)	Aghayeva et al. (2005)
	CBS125439	Soil	USA	FN546962	FN547381	Madrid et al. (2010)	Madrid et al. (2010)
	CBS125442	Soil	Spain	FN546961	FN547379	Madrid et al. (2010)	Madrid et al. (2010)
S. curviconia	CBS 541.84	*Pinus radiata*	Chile	KC113234	–	This study	–
S. globosa	CBS 130105 (FMR 8597)	Clinical strain, human sporotrichosis	Spain	FN549904	AM116964	Madrid et al. (2010)	Marimon et al. (2006)
	CBS 130104 (FMR 8595)	Clinical strain, human sporotrichosis	Spain	KC113225	AM116959	This study	Marimon et al. (2006)
	CBS 130116 (FMR 8598)	Clinical strain, human sporotrichosis	Spain	KC113226	AM116962	This study	Marimon et al. (2006)
	CBS 129720 (zx 18715)	Clinical strain, human sporotrichosis	China	KC113227	–	This study	–
	KMU 3311	Clinical strain, human sporotrichosis	Japan	AB122042	–	Watanabe et al. (2004)	–
	KMU 3360	Clinical strain, human sporotrichosis	Japan	AB122043	–	Watanabe et al. (2004)	–
	KMU 2052 (Duke 3751)	Clinical strain, human sporotrichosis	USA	AB089138	–	Watanabe et al. (2004)	–
	KMU 3993	Clinical strain, human sporotrichosis	South Africa	AB128006	–	–	–
	KMU 3509	Clinical strain, human sporotrichosis	Japan	AB128007	–	–	–
	CBS 130115 (FMR 8596)	Clinical strain, human sporotrichosis	Spain	KC113228	AM116963	This study	Marimon et al. (2006)
	CBS 130117 (FMR 9022)	Clinical strain, human sporotrichosis	Japan	KC113229	–	This study	–
	CBS 120340^{T} (FMR 8600)	Clinical strain, human sporotrichosis	Spain	KC113229	AM116966	Madrid et al. (2010)	Marimon et al. (2006)
	CBS 129725 (zx 17518)	Clinical strain, human sporotrichosis	China	KC113230	–	This study	–
	CBS 129724 (zx 17634)	Clinical strain, human sporotrichosis	China	KC113231	–	This study	–
	CBS 129722 (zx 18072)	Clinical strain, human sporotrichosis	China	KC113232	–	This study	–
	SUMS0382	–	China	FJ011549	–	–	–
	SUMS0383	–	China	FJ011550	–	–	–
	CBS 132922	Clinical strain, human sporotrichosis	Brazil	JF811336	–	Fernandes et al. (2013)	–
S. humicola	CBS 118129^{T}(CMW7618)	Soil	South Africa	AF484472	EF139100	de Beer et al. (2003)	de Meyer et al. (2008)
	CBS 120256 (CMW7617, MRC6963)	Soil	South Africa	AF484471	EF139099	de Beer et al. (2003)	de Meyer et al. (2008)

Table 1 (continued)

Species	CBS number, cross reference	Source	Origin	Genbank		ITS Reference	BT2 Reference
				ITS	BT2		
	CBS 182.63 (MUCL8061)	Soil	Netherlands	KC113233	EF139111	de Meyer et al. (2008)	de Meyer et al. (2008)
S. inflata	CBS 841.73 (CMW12535)	Soil	Chile	AY495431	AY495442	Aghayeva et al. (2005)	Aghayeva et al. (2005)
	CBS 239.68^{T} (CMW12527)	Wheat-field soil	Germany	AY495426	AY495437	Aghayeva et al. (2005)	Aghayeva et al. (2005)
	CMW 12528	*Lilium* sp.	Netherlands	AY495427	–	Aghayeva et al. (2005)	Aghayeva et al. (2005)
S.lignivora	CBS 119148^{T} (CMW 18600)	Utility poles	South Africa	EF127890	EF139104	de Meyer et al. (2008)	de Meyer et al. (2008)
	CBS 119149 (CMW 18601)	*Eucalyptus* wood pole	South Africa	EF127891	EF139105	de Meyer et al. (2008)	de Meyer et al. (2008)
	CBS 119147 (CMW 18599)	*Eucalyptus* wood pole	South Africa	EF127889	EF139103	de Meyer et al. (2008)	de Meyer et al. (2008)
	CMW 18597	*Eucalyptus* wood pole	South Africa	EF127887	EF139101	de Meyer et al. (2008)	de Meyer et al. (2008)
	CBS 119146 (CMW 18598)	*Eucalyptus* wood pole	South Africa	EF127888	EF139102	de Meyer et al. (2008)	de Meyer et al. (2008)
S. luriei	CBS 937.72^{T}	Clinical strain, human sporotrichosis	South Africa	AB128012	AM747289	–	Marimon et al. (2008)
S.mexicana	CBS 120341^{T}	Environmental	Mexico	FN549906	AM498344	Madrid et al. (2010)	Marimon et al. (2008)
S. nivea	CBS 131.56^{T} (CMW 17209)	*Stemonitis fusca*	Japan	EF127880	EF139110	de Meyer et al. (2008)	de Meyer et al. (2008)
S. pallida	CMW 7613	Clinical strain, human sporotrichosis	South Africa	AF484470	–	de Beer et al. (2003)	–
	CBS 150.87 ^{T} (CMW 17168)	Sediment in water purification plant	Germany	EF127879	EF139109	de Meyer et al. (2008)	de Meyer et al. (2008)
S. schenckii	CBS 130098 (FMR8371, IHEM15511)	Clinical strain, human sporotrichosis	Peru	KC113215	AM116917	This study	Marimon et al. (2006)
	KMU 4040	Clinical strain, human sporotrichosis	Brazil	AB128005	–	–	–
	KMU 3620	Clinical strain, human sporotrichosis	Venezuela	AB128001	–	–	–
	KMU 3944	Clinical strain, human sporotrichosis	South Africa	AB128002	–	–	–
	KMU 2500	Clinical strain, human sporotrichosis	Japan	AB089139	–	Watanabe et al. (2004)	–
	CBS 130009	Clinical strain, human sporotrichosis	Italy	KC113216	–	This study	–
	CBS 130100	Clinical strain, human sporotrichosis	Argentina	KC113217	–	This study	–
	CBS 130097 (FMR8364, IHEM15477)	Clinical strain, human sporotrichosis	Bolivia	KC113218	AM116916	This study	Marimon et al. (2006)
	CBS 130111 (FMR 8362)	Clinical strain, human sporotrichosis	Colombia	KC113219	–	This study	–
	KMU 3492	Clinical strain, human sporotrichosis	USA	AB122051	–	Watanabe et al. (2004)	–
	KMU 2285	Clinical strain, human sporotrichosis	Japan	AB127999	–	–	–
	ATCC 10268	Clinical strain, human sporotrichosis	USA	AB122038	–	Watanabe et al. (2004)	–
	CBS 938.72	Clinical strain, human sporotrichosis	France	KC113220	–	This study	–
	KMU 2687	Clinical strain, human sporotrichosis	Japan	AB122044	–	Watanabe et al. (2004)	–
	KMU 4011	Clinical strain, human sporotrichosis	Mexico	AB128008	–	–	–
	KMU 3998	Clinical strain, human sporotrichosis	South Africa	AB122052	–	Watanabe et al. (2004)	–
	KMU 4012	Clinical strain, human sporotrichosis	Mexico	AB128003	–	–	–
	KMU 4014	Clinical strain, human sporotrichosis	Mexico	AB128004	–	–	–
	CBS 130114 (FMR 8369)	Clinical strain, human sporotrichosis	Peru	KC113221	AM116930	This study	Marimon et al. (2006)

Table 1 (continued)

Species	CBS number, cross reference	Source	Origin	Genbank		ITS Reference	BT2 Reference
				ITS	BT2		
	CBS 130103 (FMR 8677)	Clinical strain, human sporotrichosis	Argentina	KC113222	AM116915	This study	Marimon et al. (2006)
	CBS 130112 (FMR 8365)	Clinical strain, human sporotrichosis	Peru	KC113223	AM116929	This study	Marimon et al. (2006)
	CBS 130101 (FMR 8604)	Clinical strain, human sporotrichosis	Peru	KC113224	AM116914	This study	Marimon et al. (2006)
	CBS 117842 (CMW 7614)	Clinical strain, human sporotrichosis	South Africa	AY280495	AY280477	Aghayeva et al. (2004)	Aghayeva et al. (2004)
	CMW 7615	Clinical strain, human sporotrichosis	South Africa	AY280496	AY280478	Aghayeva et al. (2004)	Aghayeva et al. (2004)
	CMW 7612	Clinical strain, human sporotrichosis	South Africa	AY280494	AY280476	Aghayeva et al. (2004)	Aghayeva et al. (2004)
	CMW 5681 (MRC 6864)	Clinical strain, human sporotrichosis	South Africa	EF127886	EF139107	de Meyer et al. (2008)	de Meyer et al. (2008)
	CMW 7611	Clinical strain, human sporotrichosis	South Africa	AF484469	–	de Beer et al. (2003)	–
	KMU 2286	Clinical strain, human sporotrichosis	Japan	AB122039	–	Watanabe et al. (2004)	–
	KMU 3486	Clinical strain, human sporotrichosis	USA	AB122045	–	Watanabe et al. (2004)	–
	KMU 3598	Clinical strain, human sporotrichosis	Costa Rica	AB128010	–	–	–
	CMW 7132	Clinical strain, human sporotrichosis	South Africa	AF484467	–	de Beer et al. (2003)	–
	KMU 3655	Clinical strain, human sporotrichosis	Argentina	AB122048	–	Watanabe et al. (2004)	–
	KMU 3940	Clinical strain, human sporotrichosis	South Africa	AB128011	–	–	–
	KMU 3114	Clinical strain, human sporotrichosis	Netherlands	AB128000	–	–	–
	CBS 359.36^{T}	Clinical strain, human sporotrichosis	USA	FJ545232	AM116911	-	Marimon et al. (2006)
	KMU 3504	Clinical strain, human sporotrichosis	USA	AB122046	–	Watanabe et al. (2004)	–
S.stylites	CBS 115869 (CMW 14544)	wood pole	South Africa	EF127884	EF139097	de Meyer et al. (2008)	de Meyer et al. (2008)
	CBS 118848 (CMW 14543)	wood pole	South Africa	EF127883	EF139096	de Meyer et al. (2008)	de Meyer et al. (2008)
	CMW 7133	*Rosa sp*	South Africa	AF484468	EF139098	de Beer et al. (2003)	de Meyer et al. (2008)
	CBS 115872 (CMW 14542)	wood pole	South Africa	EF127882	EF139095	de Meyer et al. (2008)	de Meyer et al. (2008)
	CBS 115868 (CMW14541)	wood pole	South Africa	EF127881	EF139094	de Meyer et al. (2008)	de Meyer et al. (2008)
S.variecibatus	CBS 121962 (CMW 2543)	Eucalyptus sp.	South Africa	DQ821567	DQ821572	Roets et al. (2008)	Roets et al. (2008)
	CBS 121960 (CMW 23060)	Protea longifolia	South Africa	DQ821569	DQ821573	Roets et al. (2008)	Roets et al. (2008)
	CBS 121961 (CMW 23051)	*Trichouropoda* sp. from *Protea repens*	South Africa	DQ821568	DQ821539	Roets et al. (2008)	Roets et al. (2008)

PCR kit (Roche, Mannheim, Germany). Both strands of the PCR fragments were sequenced with the above-mentioned primers. The ABI PrismH Big DyeTM Terminator v. 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) was used for sequencing PCR. Sequences were determined with an ABI PRISM™ 3,100 Genetic Analyzer (Applied Biosystems). DNA sequences of opposite strands were edited with Sequence Navigator version 1.0.1 (Applied Biosystems). All sequences were aligned with MAFFT v. 5.667 (Katoh et al. 2002).

DNA sequence analyses

In order to evaluate the global ITS diversity in the *S. schenckii* complex we collected nucleotide sequences from *Sporothrix*/*Ophiostoma* isolates from different regions of the world from GenBank. Methods used as well as the number of sequences retrieved in the search are exemplified in the Supplementary Fig. 1. Phylogenetic analysis included sequences previously published in the literature and in GenBank (Table 1) as well as newly generated sequences originated from clinical isolates of *Sporothrix* and other closely related environmental *Ophiostoma* and *Sporothrix* species. Phylogenetic analyses were conducted with MEGA 5 (Tamura et al. 2011) with Maximum Likelihood and Neighbor-joining methods. Evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) with a discrete Gamma distribution for the ITS dataset and Tamura 3-parameter method (Tamura 1992) for *BT2* dataset, both using 1,000 bootstrap replicates (Felsenstein 1985).

Results

In the present study we used a Boolean search method to retrieve *Sporothrix* sequences deposited in GenBank. Using '*Sporothrix*' as a query we were able to retrieve approximately 907 entries. Using an exclusive search strategy, we recovered approximately 130 nucleotide sequences that matched the ITS region (Supplementary Fig. 1). From these entries, only a few sequences were long enough (>500 bp including ITS1 + 5.8S + ITS2) to be used for confident alignment.

A final alignment was created with 124 sequences, including 25 and 99 sequences of *Ophiostoma* and *Sporothrix*, respectively. Aligned ITS sequences were 637 bp long, including 374 invariable characters, 213 variable parsimony-informative (33.4 %), and 42 singletons. Positions containing gaps and missing data were eliminated. *Indel* regions were evaluated considering the sequence FJ545232 from the type strain of *S. schenckii* (CBS 359.36) from Maryland, USA (Fig. 1a). The variation within the phylogenetically related species of clinical interest (68 sequences), including *S. schenckii*, *S. brasiliensis* and *S. globosa* is shown in Fig. 1b. A substantial number of 17 polymorphic sites was noted differentiating the species *S. brasiliensis* from its sister taxon *S. schenckii* (14 of them were parsimony-informative). The ITS1 region had a higher degree of mutations than ITS2 (ITS1/ITS2 ratio=2,4).

An ITS phylogenetic tree was constructed using Maximum likelihood (model K2-P + G) with 1,000 bootstrap replications (Fig. 2). The 124 sequences were distributed into 30 taxa described in previous studies (de Beer et al. 2003; Zhou et al. 2006; Zipfel et al. 2006; Roets et al. 2006, 2008; de Meyer et al. 2008; Madrid et al. 2010). An unambiguous separation between species of clinical importance and strictly environmental species was observed, with strains from human and animal sources being concentrated in the clades *S. brasiliensis*, *S. schenckii*, *S. globosa* and *S. luriei* (A; Fig. 2).

The geographical distribution of isolates in the clinical clade varied with the species. The clade identified as *S. brasiliensis* (B) had a high incidence in South America, being restricted to Brazil (Fig. 2). The phylogenetic group identified here as *S. schenckii s.str.* was subdivided into two clusters. A first group (C) adjacent to *S. brasiliensis* prevailed in the Americas (61 %), followed by Asia (17 %), Africa (11 %) and Europe (11 %). A second set of *S. schenckii* strains (D) harboring the type strain CBS 359.36 prevailed in the Americas (50 %), followed by Africa (38 %), Asia (6 %) and Europe (6 %). *Sporothrix globosa* (E) is present with high frequency in Asia (56 %) and Europe (28 %), followed by the Americas (11 %) and Africa (5 %).

In the remaining tree, *Sporothrix* species were flanked by *Ophiostoma* species which were mainly derived from soil, plants or found in association with bark beetles. Despite the good taxonomic resolution achieved for clinical species, in the environmental clade some taxa were not easily differentiated using the ITS region. *Sporothrix mexicana* was located amidst the environmental *Sporothrix* species (*S. humicola, S. stylites, S. pallida,* and *S. nivea*) which had identical ITS sequences. The same was found with several clusters of *Ophiostoma* species. *Sporothrix brunneoviolacea* and *S. lignivora* took remote positions; *S. lignivora* was used to root the tree.

In order to calibrate the ITS-based phylogeny, some isolates from each taxa evaluated previously were chosen for a second analysis using the *BT2* region (including the type strains for each species). This region was selected because it has been widely used for taxonomy of environmental *Ophiostoma*/*Sporothrix* species (Roets et al. 2006, 2008; Zipfel et al. 2006). The *BT2* complete alignment included 71 sequences (Table 1). Aligned sequences of *BT2* matched 607 characters, including 189 invariable characters, 307 variable parsimony-informative (50,5 %), and 37 singletons.

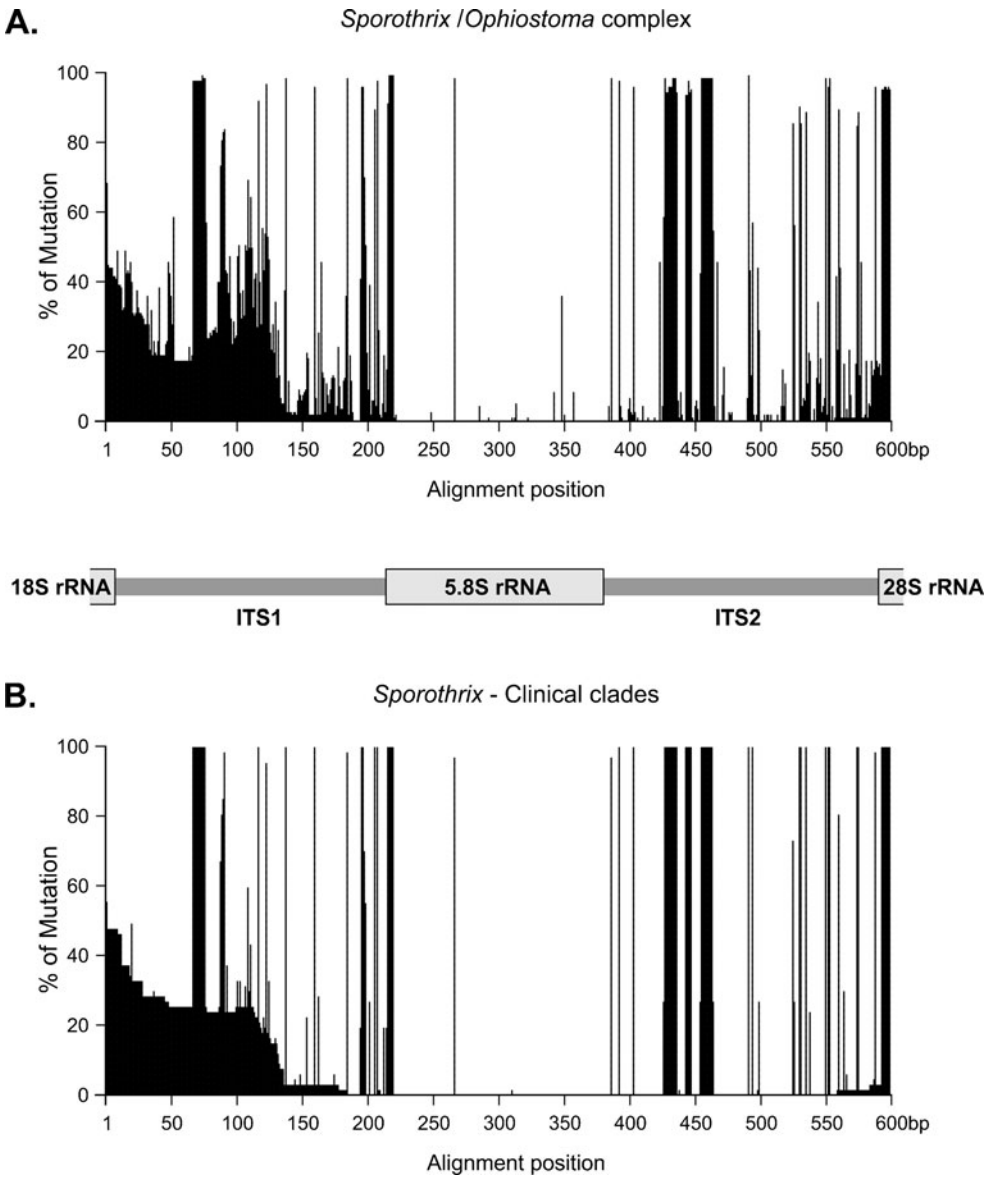


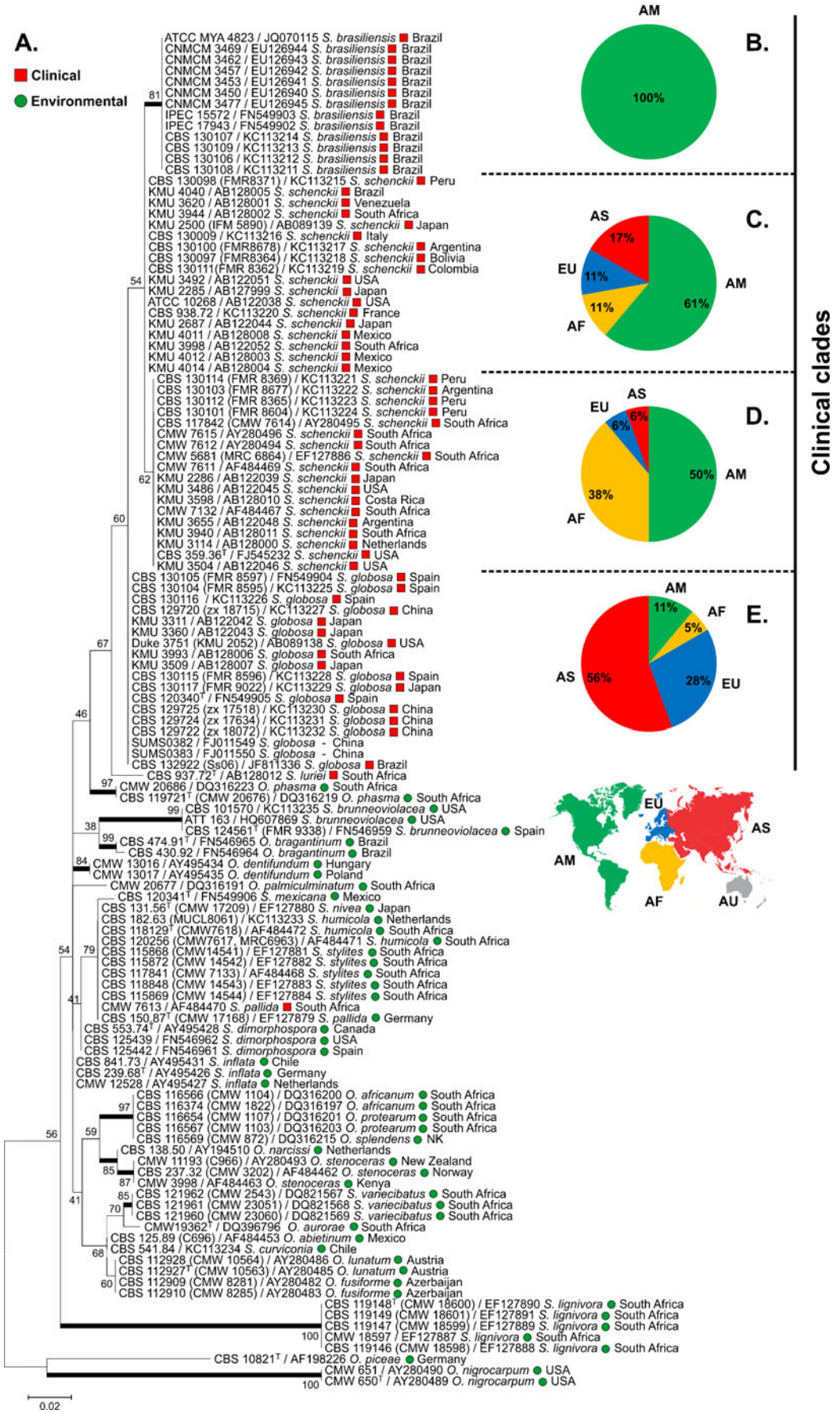
Fig. 1 Polymorphisms in ITS1/2 + 5.8S nucleotide sequences of *Sporothrix* and *Ophiostoma*. **a** Mutations at each position in the aligned sequences (n=124) including environmental and clinical isolates in S*porothrix* and *Ophiostoma* complex. **b** Sequence comparison among 68 strains in the clinical clade including *S. brasiliensis, S. schenckii* and *S. globosa*. All comparisons were done relative to *S. schenckii* type strain CBS 359.36 (FJ545232)

Positions containing gaps and missing data were eliminated. All taxa were clearly separated using this locus. A strong separation between clinical and environmental clades was observed for *BT2*, coinciding with the bipartition found in ITS. In agreement with ITS phylogeny, the environmental species *O. phasma* was the nearest taxon to the *S. schenckii* complex. This topology is in agreement with previous studies (Roets et al. 2006, 2010; Madrid et al. 2010). The topologies of trees of *BT2* and ITS showed strict correspondence, with all clinical clades being recognized using both genes.

Discussion

Our data provide a representation of the *S. schenckii* complex as it is embedded in the phylogeny of Ophiostomatales (de Beer et al. 2003; de Meyer et al. 2008). An *Ophiostoma* teleomorph has been predicted for *S. schenckii* on the basis of morphological similarity with anamorphs of e.g. *O. stenoceras* and *O. nigrocarpum* as supported by sequence data (Berbee and Taylor 1992; de Beer et al. 2003). In our analysis, *Ophiostoma phasma* (CMW 20676) from *Protea* in South Africa appears most closely related (Fig. 2a). However, *S. schenckii* is still located at relatively large distance. By combined phenotypic and molecular characters Marimon et al. (2007) introduced *S. brasiliensis, S. globosa*

Fig. 2 Phylogenetic relationships inferred from maximum likelihood ▶ analysis of ITS sequences of 124 strains belonging to *Sporothrix* and *Ophiostoma*. *Numbers* close to the branches represent indices of support based on 1,000 bootstrap replications. Branches with bootstrap support higher than 70 % are indicated in bold. *Sporothrix brasiliensis* (**b**), *S. schenckii* (**c** and **d**) and *S. globosa* (**e**) frequencies are calculated from clinical isolates collected worldwide and available at GenBank. Isolates were listed according the geographical origin from the Americas (AM), Europe (EU), Africa (AF), Asia (AS), or Australia (AU)


A.
Clinical
Environmental
ATCC MYA 4823 / JQ070115 S. brasiliensis Brazil
CNMCM 3469 / EU126944 S. brasiliensis Brazil
CNMCM 3462 / EU126943 S. brasiliensis Brazil
CNMCM 3457 / EU126942 S. brasiliensis Brazil
CNMCM 3453 / EU126941 S. brasiliensis Brazil
CNMCM 3450 / EU126940 S. brasiliensis Brazil
CNMCM 3477 / EU126945 S. brasiliensis Brazil
IPEC 15572 / FN549903 S. brasiliensis Brazil
IPEC 17943 / FN549902 S. brasiliensis Brazil
CBS 130107 / KC113214 S. brasiliensis Brazil
CBS 130109 / KC113213 S. brasiliensis Brazil
CBS 130106 / KC113212 S. brasiliensis Brazil
CBS 130108 / KC113211 S. brasiliensis Brazil
CBS 130098 (FMR8371) / KC113215 S. schenckii Peru
KMU 4040 / AB128005 S. schenckii Brazil
KMU 3620 / AB128001 S. schenckii Venezuela
KMU 3944 / AB128002 S. schenckii South Africa
KMU 2500 (IFM 5890) / AB089139 S. schenckii Japan
CBS 130009 / KC113216 S. schenckii Italy
CBS 130100 (FMR8678) / KC113217 S. schenckii Argentina
CBS 130097 (FMR8364) / KC113218 S. schenckii Bolivia
CBS 130111(FMR 8362) / KC113219 S. schenckii Colombia
KMU 3492 / AB122051 S. schenckii USA
KMU 2285 / AB127999 S. schenckii Japan
ATCC 10268 / AB122038 S. schenckii USA
CBS 938.72 / KC113220 S. schenckii France
KMU 2687 / AB122044 S. schenckii Japan
KMU 4011 / AB128008 S. schenckii Mexico
KMU 3998 / AB122052 S. schenckii South Africa
KMU 4012 / AB128003 S. schenckii Mexico
KMU 4014 / AB128004 S. schenckii Mexico
CBS 130114 (FMR 8369) / KC113221 S. schenckii Peru
CBS 130103 (FMR 8677) / KC113222 S. schenckii Argentina
CBS 130112 (FMR 8365) / KC113223 S. schenckii Peru
CBS 130101 (FMR 8604) / KC113224 S. schenckii Peru
CBS 117842 (CMW 7614) / AY280495 S. schenckii South Africa
CMW 7615 / AY280496 S. schenckii South Africa
CMW 7612 / AY280494 S. schenckii South Africa
CMW 5681 (MRC 6864) / EF127886 S. schenckii South Africa
CMW 7611 / AF484469 S. schenckii South Africa
KMU 2286 / AB122039 S. schenckii Japan
KMU 3486 / AB122045 S. schenckii USA
KMU 3598 / AB128010 S. schenckii Costa Rica
CMW 7132 / AF484467 S. schenckii South Africa
KMU 3655 / AB122048 S. schenckii Argentina
KMU 3940 / AB128011 S. schenckii South Africa
KMU 3114 / AB128000 S. schenckii Netherlands
CBS 359.36T / FJ545232 S. schenckii USA
KMU 3504 / AB122046 S. schenckii USA
CBS 130105 (FMR 8597) / FN549904 S. globosa Spain
CBS 130104 (FMR 8595) / KC113225 S. globosa Spain
CBS 130116 / KC113226 S. globosa Spain
CBS 129720 (zx 18715) / KC113227 S. globosa China
KMU 3311 / AB122042 S. globosa Japan
KMU 3360 / AB122043 S. globosa Japan
Duke 3751 (KMU 2052) / AB089138 S. globosa USA
KMU 3993 / AB128006 S. globosa South Africa
KMU 3509 / AB128007 S. globosa Japan
CBS 130115 (FMR 8596) / KC113228 S. globosa Spain
CBS 130117 (FMR 9022) / KC113229 S. globosa Japan
CBS 120340T / FN549905 S. globosa Spain
CBS 129725 (zx 17518) / KC113230 S. globosa China
CBS 129724 (zx 17634) / KC113231 S. globosa China
CBS 129722 (zx 18072) / KC113232 S. globosa China
SUMS0382 / FJ011549 S. globosa - China
SUMS0383 / FJ011550 S. globosa - China
CBS 132922 (Ss06) / JF811336 S. globosa Brazil
CBS 937.72T / AB128012 S. luriei South Africa
CMW 20686 / DQ316223 O. phasma South Africa
CBS 119721T (CMW 20676) / DQ316219 O. phasma South Africa
CBS 101570 / KC113235 S. brunneoviolacea USA
ATT 163 / HQ607869 S. brunneoviolacea USA
CBS 124561T (FMR 9338) / FN546959 S. brunneoviolacea Spain
CBS 474.91T / FN546965 O. bragantinum Brazil
CBS 430.92 / FN546964 O. bragantinum Brazil
CMW 13016 / AY495434 O. dentifundum Hungary
CMW 13017 / AY495435 O. dentifundum Poland
CMW 20677 / DQ316191 O. palmiculminatum South Africa
CBS 120341T / FN549906 S. mexicana Mexico
CBS 131.56T (CMW 17209) / EF127880 S. nivea Japan
CBS 182.63 (MUCL8061) / KC113233 S. humicola Netherlands
CBS 118129T (CMW7618) / AF484472 S. humicola South Africa
CBS 120256 (CMW7617, MRC6963) / AF484471 S. humicola South Africa
CBS 115868 (CMW14541) / EF127881 S. stylites South Africa
CBS 115872 (CMW 14542) / EF127882 S. stylites South Africa
CBS 117841 (CMW 7133) / AF484468 S. stylites South Africa
CBS 118848 (CMW 14543) / EF127883 S. stylites South Africa
CBS 115869 (CMW 14544) / EF127884 S. stylites South Africa
CMW 7613 / AF484470 S. pallida South Africa
CBS 150.87T (CMW 17168) / EF127879 S. pallida Germany
CBS 553.74T / AY495428 S. dimorphospora Canada
CBS 125439 / FN546962 S. dimorphospora USA
CBS 125442 / FN546961 S. dimorphospora Spain
CBS 841.73 / AY495431 S. inflata Chile
CBS 239.68T / AY495426 S. inflata Germany
CMW 12528 / AY495427 S. inflata Netherlands
CBS 116566 (CMW 1104) / DQ316200 O. africanum South Africa
CBS 116374 (CMW 1822) / DQ316197 O. africanum South Africa
CBS 116654 (CMW 1107) / DQ316201 O. protearum South Africa
CBS 116567 (CMW 1103) / DQ316203 O. protearum South Africa
CBS 116569 (CMW 872) / DQ316215 O. splendens NK
CBS 138.50 / AY194510 O. narcissi Netherlands
CMW 11193 (C966) / AY280493 O. stenoceras New Zealand
CBS 237.32 (CMW 3202) / AF484462 O. stenoceras Norway
CMW 3998 / AF484463 O. stenoceras Kenya
CBS 121962 (CMW 2543) / DQ821567 S. variecibatus South Africa
CBS 121961 (CMW 23051) / DQ821568 S. variecibatus South Africa
CBS 121960 (CMW 23060) / DQ821569 S. variecibatus South Africa
CMW19362T / DQ396796 O. aurorae South Africa
CBS 125.89 (C696) / AF484453 O. abietinum Mexico
CBS 541.84 / KC113234 S. curviconia Chile
CBS 112928 (CMW 10564) / AY280486 O. lunatum Austria
CBS 112927T (CMW 10563) / AY280485 O. lunatum Austria
CBS 112909 (CMW 8281) / AY280482 O. fusiforme Azerbaijan
CBS 112910 (CMW 8285) / AY280483 O. fusiforme Azerbaijan
CBS 119148T (CMW 18600) / EF127890 S. lignivora South Africa
CBS 119149 (CMW 18601) / EF127891 S. lignivora South Africa
CBS 119147 (CMW 18599) / EF127889 S. lignivora South Africa
CMW 18597 / EF127887 S. lignivora South Africa
CBS 119146 (CMW 18598) / EF127888 S. lignivora South Africa
CBS 10821T / AF198226 O. piceae Germany
CMW 651 / AY280490 O. nigrocarpum USA
CMW 650T / AY280489 O. nigrocarpum USA
0.02
B.
AM
100%
C.
AS
17%
EU
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AM
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Clinical clades

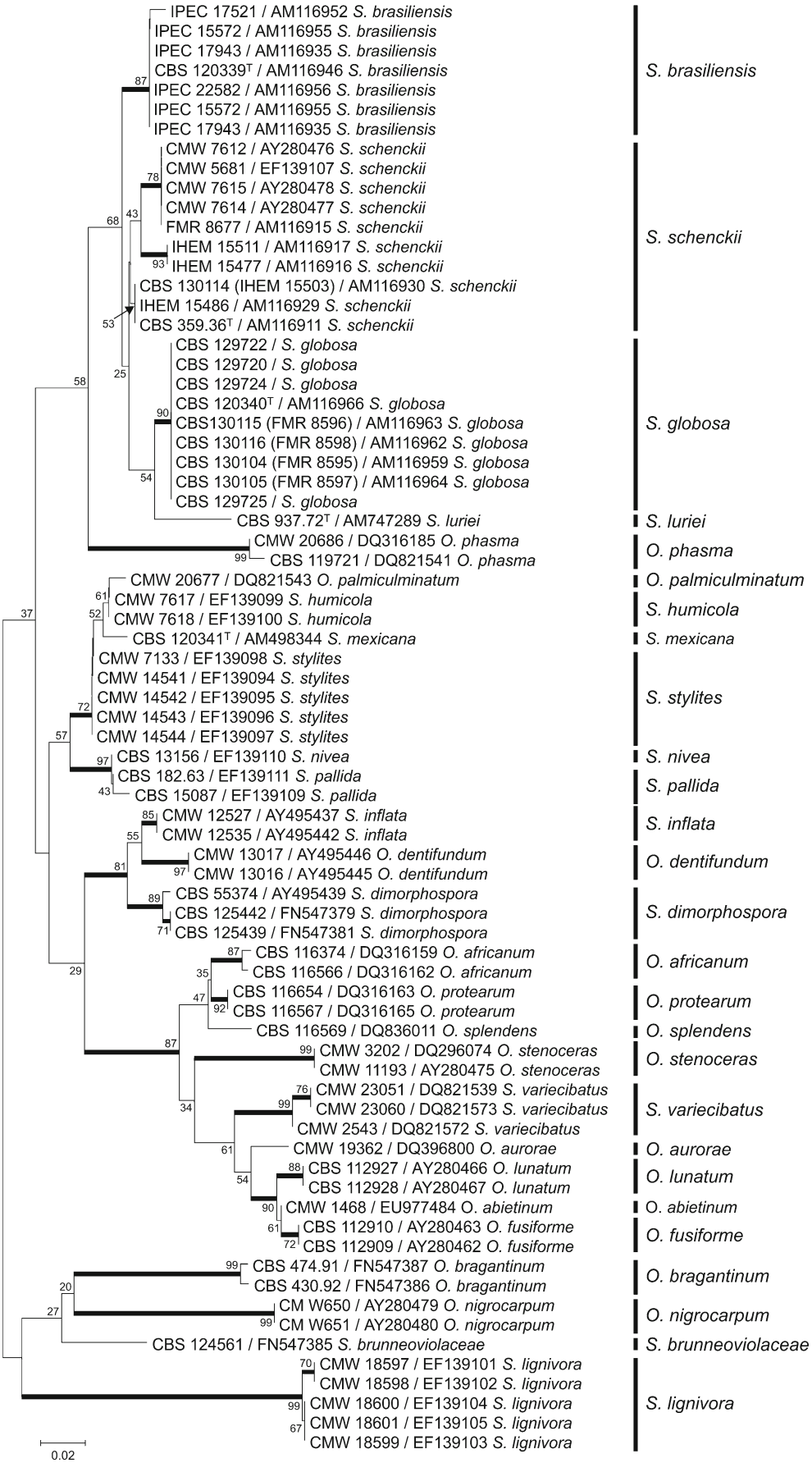

Fig. 3 Phylogenetic relationships of *Sporothrix* and *Ophiostoma* inferred from β-tubulin sequences by Neighbor-joining algorithm based on the Tamura 3-parameter model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Bootstrap support values >80 are indicated in bold). *GenBank accessions numbers* are indicated next to strain code

and *S. mexicana* as new species, next to *S. luriei*, a variety of *S. schenckii* which was attributed species status. Earlier described species such as *S. pallida* and *S. inflata* were confirmed to be distinct taxa. Although Marimon et al. (2007) made a substantial contribution towards understanding the relationships within the species complex, their study was limited mainly to clinical isolates from few geographical origins. The present study expands the investigation of *Sporothrix* species in ecological origins and global representation of strains analyzed. In our expanded comparison it is apparent that *S. mexicana* is remote, being a member of the *S. pallida* complex (Fig. 2). This species presents a mild potential pathogenicity to humans (Rodrigues et al. 2012), which is exceptional outside the *S. schenckii* complex.

The *S. schenckii* complex is presently restricted to four species: *S. schenckii s.str., S brasiliensis, S. globosa* and *S. luriei*, which deviate not only phylogenetically from the remainder of Ophiostomatales, but also by their virulence to mammals. Species distinction within the complex is presently based on partial calmodulin sequences (Marimon et al. 2007; Oliveira et al. 2011; Romeo et al. 2011). The topology of the *BT2* tree (Fig. 3) is broadly concordant with that of the ITS tree (Fig. 2), and both are essentially similar to that derived from partial calmodulin sequences in Oliveira et al. (2011) and Romeo et al. (2011). Several of the ITS clades, including the *S. schenckii* clade are statistically supported with high bootstrap values (Fig. 2). Other groups of species such as those around *S. pallida* have identical ITS sequences, and similar clusters of closely related species are noted e.g. with *Ophiostoma proteae* and *O. africanum*.

In the highly supported, derived clade of *S. schenckii* and relatives the ITS differences are large enough to distinguish all four presently recognized taxa using this widely applied barcoding gene. Three of the four species of this group are known from pseudoepidemics: *S. brasiliensis* in Brazil (Rodrigues et al. 2012), *S. schenckii s.l.* in South Africa (Vismer and Hull 1997), and *S. globosa* in China (Han et al. 2006; Li et al. 2007; Zhang and Lin 2008; Mei et al. 2011; Wang et al. 2012). Although *Sporothrix* species are primarily environmental, their traumatic inoculation into human hosts e.g. by wood splinters from pine wood (South Africa) or from scratches of cat paws in Brazil (Schubach et al. 2005, 2008) is highly efficient. This is quite a remarkable feature in the fungal Kingdom, where (pseudo)epidemics are exceptional. Outside the *S. schenckii* complex, only accidental, unlinked cases are known, e.g. *O. stenoceras* (Summerbell 1993), *O. piceae* (Bommer et al. 2009), and *S. mexicana* (Rodrigues et al. 2012). Isolate CMW 7613, previously identified as *S. schenckii* by de Beer et al. (2003) grouped within the *S. pallida* complex, a typically environmental clade, although this isolate was derived from a human case of sporotrichosis in South Africa. These examples show that mammal-pathogenicity also occurs outside the *S. schenckii* complex, but it remains highly exceptional with scattered cases. In the *S. schenckii* complex a high degree of virulence is constitutive, and in this sense the group deviates considerably from the remaining Ophiostomatales. It is recommended that the group remains separate, and that it is not merged with *Ophiostoma*.

Utilizing length polymorphisms within the ITS region we were able to detect epidemiological differences among clinical *Sporothrix* strains. In agreement with previous studies (Marimon et al. 2006, 2007), *S. brasiliensis* remains restricted to the Brazilian territory (Fig. 2). Most of the sequences representing the first group (C) of *S. schenckii* are predominant in the Americas and Asia, whereas those of the second group (D) are present in the Americas followed by Africa. Europe has a low incidence of sporotrichosis. Surprisingly the pathogenic species *S. globosa* (E) is predominant in Asia and Europe, but rare in the Americas and Africa. This is in agreement with Madrid et al. (2009) that show *S. schenckii* is more common in Americas than *S. globosa*. Figure 2 also shows that strains originating from a restricted geographic area mostly do not constitute monophyletic lineages. For example, strains from South Africa scattered in at least 3 major clades, and the same was found for the strains from Japan. Our increased geographic sampling for the ITS dataset did not affect delimitation success in the clinical clade. Unfortunately we were unable to compare strains and sequences from Australian epidemics of sporotrichosis; *S. schenckii* was identified as the causal agent of the Australian epidemics using morphological characters and the pulsed-field gel electrophoresis (PFGE) technique (O'Reilly and Altman 2006; Feeney et al. 2007).

ITS is the most widely applied gene for routine identification, and has been recommended as a fungal barcoding gene (Seifert 2009; Schoch et al. 2012; Toju et al. 2012). The four current species of the complex are all distinguishable by fixed mutations. The smallest barcoding gap is between *S. schenckii* and *S. brasiliensis,* at a minimum distance of 4.44 % and mean interspecific divergence of 0.0079. We conclude that ITS is sufficient for routine species distinction of all clinically relevant *Sporothrix*-like species including the occasional agents in Ophiostomatales that are remote from the *S. schenckii* complex.

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