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Taxonomy and phylogeny of new wood- and soil-inhabiting *Sporothrix* species in the *Ophiostoma stenoceras-Sporothrix schenckii* complex

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Abstract: *Sporothrix*, one of the anamorph genera of *Ophiostoma*, includes the important human pathogen *S. schenckii* and various fungi associated with insects and sap stain of wood. A survey of fungi from wood utility poles in South Africa yielded two distinct groups of *Sporothrix* isolates from different geographical areas. DNA sequence and morphological data derived in this study showed that isolates in these groups represent two novel species in the *S. schenckii-O. stenoceras* species complex. A new species isolated from pine poles and rosebush wood and phylogenetically closely related to *S. pallida* is described here as *Sporothrix stylites*. Phylogenetic analyses also confirmed the synonymy of *S. albicans* and *S. nivea* with *S. pallida*. *Sporothrix stylites* and *S. pallida* also are related closely to the isolates from soil, previously treated as ''environmental'' isolates of *S. schenckii*. Soil isolates are clearly distinct from human isolates of *S. schenckii*. We describe the former here as *Sporothrix humicola*. The isolates from eucalypt poles group peripheral to most other species in the *S. schenckii-O. stenoceras* complex and are newly described as *Sporothrix lignivora*. Phylogenetic analyses of sequences of isolates from soil and wood together with those of clinical isolates showed that the human-pathogenic strains form an aggregate of several cryptic species.

Key words: human pathogen, Ophiostomatales, sporotrichosis

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INTRODUCTION

Sporothrix Hektoen & C.F. Perkins is one of the anamorph genera associated with the ascomycete genus *Ophiostoma* Syd. & P. Syd. (Zipfel et al 2006). Species of *Ophiostoma* are well known symbionts of bark beetles (Six 2003, Kirisits 2004) and as the causal agents of sap stain in freshly cut wood (Seifert 1993). Genus *Sporothrix* however is most commonly recognized on account of its type species *S. schenckii* Hektoen & C.F. Perkins being an important human pathogen (de Hoog 1993). Although no teleomorph is known for *S. schenckii*, ribosomal DNA sequences have confirmed the long-standing biosystematic judgment that it is related phylogenetically to genus *Ophiostoma* (*Ophiostomatales*) and specifically to the cosmopolitan, normally nonpathogenic *O. stenoceras* (Berbee and Taylor 1992, Summerbell et al 1993, de Beer et al 2003).

Sporothrix schenckii grows saprotrophically on wood, peat moss and organic litter and causes cutaneous or subcutaneous infections in humans and other mammals when traumatically implanted under the skin, for example by scratches caused by infested splinters (Travassos and Lloyd 1980, Summerbell et al 1993). Sporotrichosis, the disease it causes, has been reported worldwide (Travassos and Lloyd 1980, Watanabe et al 2004). In South Africa the disease was reported first in 1914 (Pijper and Pullinger 1927). During the 1940s sporotrichosis reached epidemic proportions in South African gold mines where untreated timbers were used as props in tunnels (Brown et al 1947). The disease incidence in mines has decreased since preservative treatment of timbers became a standard practice (Helm and Berman 1947). However since the late 1980s increasing numbers of reports have documented severe cases of sporotrichosis in HIV-positive patients, not only in tropical and subtropical Africa (Callens et al 2006) but also in North America (Shaw et al 1989, Heller and Fuhrer 1991), South America (Rocha et al 2001) and Europe (Hardman et al 2005). Although sporotrichosis is not among the most common fungal

infections in immunocompromised patients it is difficult to eliminate when it does occur (Hardman et al 2005).

Attempts to study the epidemiology of sporotrichosis have long been confounded by the environmental co-occurrence of pathogenic *S. schenckii* isolates with morphologically similar isolates that prove to be nonpathogenic in animal tests (Howard and Orr 1963, Mackinnon et al 1969, Mariat 1975, Cooper et al 1992, Ishizaki et al 2002, de Beer et al 2003). The nonpathogenic isolates, often from wood, soil or peat moss suspected to be sources of inoculum in sporotrichosis outbreaks, were included along with the *S. schenckii*-like anamorphs of *Ophiostoma stenoceras* in a broad species concept for *S. schenckii* proposed by de Hoog (1974). However both earlier (Mackinnon et al 1969) and later researchers (Mariat 1975, Cooper et al 1992, Dixon et al 1992) concurred that fresh clinical and environmental isolates of the pathogenic ''true'' *S. schenckii* were distinguishable from the nonpathogens, including *O. stenoceras* anamorphs as well as isolates not forming a teleomorph, by their formation of melanized ''secondary conidia'' on the substrate mycelium. Nonpathogens were confirmed further as distinct from *S. schenckii* via analyses of their mitochondrial (Suzuki et al 1988, Ishizaki et al 2002) and whole-cell (Cooper et al 1992) DNA restriction patterns as well as their nuclear ribosomal DNA internal transcribed spacer (ITS) sequences (de Beer et al 2003). The correct naming of the nonpathogenic anamorphic species isolated in such surveys has long remained an outstanding problem, as noted by Summerbell et al (1993).

The development of DNA-based techniques to study relationships among confirmed pathogenic *S. schenckii* isolates has been rapid. The techniques applied most widely to distinguish groups within *S. schenckii* are RFLP analyses of the mtDNA (Takeda et al 1991; Ishizaki et al 1996, 1998, 2000; Lin et al 1999; Mora-Cabrera et al 2001; Arenas et al 2007) and ITS regions (Watanabe et al 2004). More recently AFLP (Neyra et al 2005), pulsed-field gel electrophoresis (PFGE) (O'Reilly and Altmann 2006) and a multigene phylogeny employing chitin synthase, β-tubulin and calmodulin sequences (Marimon et al 2006) confirmed the existence of several genotypes among clinical *S. schenckii* isolates. In a subsequent study Marimon et al (2007) described three of these groups as new species based primarily on differences in calmodulin sequences. Most of these studies focused on collections of isolates from specific geographical regions and did not include other closely related *Ophiostoma* or *Sporothrix* species. The failure to include key reference isolates and the use of different techniques have made it difficult to compare among or link groups of isolates revealed in the different studies.

A survey was conducted to determine the causal agents of fungal decay on wood utility poles in South Africa (de Meyer 2007). Several isolates broadly resembling *S. schenckii* were obtained from three of the six geographical areas included in the survey. The objectives of the present study were to identify these *Sporothrix* isolates and to determine their phylogenetic relationships to clinical and environmental isolates of *S. schenckii*, as well as other known *Sporothrix* and *Ophiostoma* species. Various poorly known *Sporothrix* names represented by ex-type isolates in the CBS collection (Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands) were included in the study to determine their phylogenetic status with respect to *S. schenckii* as well as the South African isolates. Comparisons were done at genus level with nuclear large subunit ribosomal DNA (LSU) data and at species level using ITS and partial β-tubulin sequences.

MATERIALS AND METHODS

Isolates examined.—All freshly collected isolates originated from South Africa (TABLE I). Eight of the environmental isolates examined came from wood utility poles, two from soil and one from a rose bush stem. One clinical isolate of *S. schenckii* also was included. For reference purposes the ex-type isolates of *Sporothrix pallida* (Tubaki) Matsush., *S. albicans* Saksena and *S. nivea* Kreisel & Shauer were included. All South African isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative strains also have been deposited at the CBS (Centraalbureau voor Schimmelcultures), Utrecht, the Netherlands.

DNA extraction and PCR amplification.—DNA extraction was performed with PrepMan™ Ultra (Applied Biosystems, Foster City, California) as described by Aghayeva et al (2004). The 3′ end of the small subunit (SSU) rDNA gene, the ITS regions 1 and 2, including the 5.8S rDNA gene as well as the 5′ end of the LSU, were amplified with primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al 1990). The PCR reaction mixture consisted of 2.6 U Super-Therm Polymerase (Hoffman-LaRoche), 5 µL PCR reaction buffer, 3 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.2 mM of each primer (total volume 50 µL). PCR conditions were 2 min at 95 C followed by 40 cycles of 30 s at 95 C, 30 s at 52 C and 1 min at 72 C, followed by one cycle of 8 min at 72 C. A fragment of the LSU gene was amplified with primers LR5 and LROR (http://www.biology.duke.edu/fungi/mycolab/primers.htm) using the same reaction mixture and PCR conditions as for ITS.

The partial β-tubulin gene was amplified with primers Bt2a and Bt2b (Glass and Donaldson 1995). The reaction mixture was the same as for the ITS regions. PCR conditions

TABLE I. *Sporothrix* isolates considered in this study

Name	CMW No.	Other numbers	Herbarium No.	Isolated from	Collector or supplier	GenBank			Origin
						ITS	β-tubulin	LSU	
S. stylites	14541	CBS 115868	PREM 59276	Pine utility poles	EM de Meyer	EF127881	EF139094		Plettenberg Bay, SA
	14542	CBS 115872	PREM 59277	Pine utility poles	EM de Meyer	EF127882	EF139095		Plettenberg Bay, SA
	14543	CBS 118848	PREM 59278[T]	Pine utility poles	EM de Meyer	EF127883	EF139096	EF139115	Plettenberg Bay, SA
	14544	CBS 115869	PREM 59279	Pine utility poles	EM de Meyer	EF127884	EF139097	EF139116	Plettenberg Bay, SA
	7133		PREM 59280	*Rosa* sp.	ZW de Beer	AF484468	EF139098		SA
S. humicola	7617	CBS 120256; MRC 6963	PREM 59281	Soil	HF Vismer	AF484471	EF139099	EF139113	SA
	7618	CBS 118129; MRC 6965	PREM 59282[T]	Soil	HF Vismer	AF484472	EF139100	EF139114	SA
S. lignivora	18601	CBS 119149	PREM 59283	Eucalyptus utility poles	EM de Meyer	EF127891	EF139105	EF139120	Stellenbosch, SA
	18600	CBS 119148	PREM 59284[T]	Eucalyptus utility poles	EM de Meyer	EF127890	EF139104	EF139119	Stellenbosch, SA
	18599	CBS 119147	PREM 59285	Eucalyptus utility poles	EM de Meyer	EF127889	EF139103		Stellenbosch, SA
	18598	CBS 119146	PREM 59286	Eucalyptus utility poles	EM de Meyer	EF127888	EF139102	EF139118	St Lucia, SA
	18597		PREM 59287	Eucalyptus utility poles	EM de Meyer	EF127887	EF139101	EF139117	St Lucia, SA
S. schenckii	5681	MRC 6864		Human sporotrichosis	HF Vismer	EF127886	EF139107	EF139112	SA
S. schenckii var. *luriei*	17210	CBS 937.72		Human skin	H Lurie	AB128012			SA
S. nivea	17168	CBS 150.87[ET]		Sediment in water purification plant	H Kreisel	EF127879	EF139109		Germany
S. pallida	17209	CBS 131.56[ET]		Sporophore of *Stemonitis fusca*	K Tubaki	EF127880	EF139110	EF139121	Japan
		CBS 182.63		Garden soil	JH van Emden		EF139111		Netherlands
S. albicans	17203	CBS 302.73[ET]		Soil	SB Saksena		EF139108		England

CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.
CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
PREM = National Fungal Herbarium, Pretoria, South Africa.
MRC = PROMEC Unit, Medical Research Council, Cape Town.
[T] = Type specimens.
[ET] = Ex-type cultures.

were 1 min at 94 C followed by 30 cycles of 94 C for 1 min, 50–55 C (different for different isolates) for 1 min and 72 C for 1 min. A last elongation step of 5 min at 72 C followed. The PCR products were viewed by electrophoresis on a 1% (w/v) agarose gel, stained with ethidium bromide.

PCR products were purified with the High Pure PCR kit (Roche, Mannheim, Germany). Both strands of the PCR fragments were sequenced with the above-mentioned primers as well as primers ITS2 and ITS3 for the ITS regions. The ABI Prism® Big Dye™ Terminator v.3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, California) was used for sequencing PCR. Sequences were determined with an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems). DNA sequences of opposite strands were edited with Sequence Navigator version 1.0.1 (ABI Prism, Perkin Elmer). All sequences were aligned with MAFFT v. 5.667 (Katoh et al 2002). Aligned datasets were deposited in TreeBase.

DNA sequence analyses.—For phylogenetic analyses sequences from previous studies (Aghayeva et al 2004, 2005; Watanabe et al 2004; Marimon et al 2006; Roets et al 2006a; Zipfel et al 2006; Zhou et al 2006) were obtained from GenBank. Accession numbers for these sequences are provided (FIGS. 1–3). The sequences were derived from *S. schenckii* isolates from outside South Africa and of other closely related *Ophiostoma* and *Sporothrix* species. Phylogenetic analyses were conducted with MEGA 3.1 (Kumar et al 2004). Distance analyses were done to construct neighbor joining trees with the Kimura 2-parameter model. One thousand bootstrap replicates were run to determine confidence levels of the branching points (Felsenstein 1985). For maximum parsimony analyses the CNI search option was used and 1000 bootstrap replicates were done to test for internal node support. The LSU tree was rooted with two isolates of *Grosmannia penicillata* (Grosmann) Goid., the type species of the genus *Grosmannia* Goid. (Zipfel et al 2006).

Bayesian phylogenetic inference was done in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). MrModeltest 2.2 (http://www.abc.se/~nylander/) was used to determine the appropriate substitution models, and Bayesian analyses were run 10 000 000 generations, sampled every 100 generations. Four chains and a random starting tree were used for all analyses. Burn-in was determined for each analysis with Tracer 1.4 (http://tree.bio.ed.ac.uk/software/tracer/). Posterior probabilities were obtained from Markov chain Monte Carlo (MCMC) results and Bayesian trees viewed in MEGA 3.1.

Morphology.—Cultures were grown on 2% malt extract agar (MEA: 20 g malt extract Biolab, Merck, Midrand, South Africa; 15 g agar Biolab, Merck; 1000 mL dH_2O). To study the formation of conidia and conidiogenous cells slides were prepared with the slide culture method (Riddell 1950) with some modifications. A clean block of 2% MEA was cut from a Petri dish and placed inside of the lid. The sides of the block were inoculated with the culture, a sterilized cover slide was placed over the block and the lid placed back on the dish and incubated. When the fungus had grown onto the cover slide, this was removed and placed on an object slide and fixed with lactophenol or 85% lactic acid. Fungal structures from older cultures also were mounted directly on slides to investigate the morphology of mature spores. Microscope observations were made with a Zeiss Axioscop compound microscope and photographic images captured with a Zeiss Axiocam MRc digital camera. AxioVision 3.1 (Carl Zeiss Ltd., Germany) was used for all measurements. Fifty measurements were taken of each taxonomically informative structure of the isolates chosen to represent the new species. Colony colors were defined with Rayner's (1970) charts.

Conidiogenous structures of selected isolates were examined with scanning electron microscopy. Cultures were grown on oatmeal agar 14 d. A colonized block of agar was cut out and immersed in 2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7) 1 h. The specimens were washed three times (10 min each) in 0.075 M phosphate buffer and fixed in 0.5% aqueous osmium tetroxide 1–2 h. The material was washed three times in distilled water and dehydrated (10 min each in 30%, 50%, 70%, 90%, 100%, 100% and 100% ethanol). The specimens were critical point dried, mounted on aluminium stubs and sputtered coated with gold. Specimens were examined with JSM-840 SEM (JEOL, Tokyo) at 5 kV and images captured with Frame Grabber: Image Slave for Windows v 2.14 (The Dindima Group, Ringwood, Victoria, Australia).

Cultures were grown 14 d on 2% MEA to provide inoculum for the growth comparisons in culture. A 5 mm cork borer was used to obtain circular inoculum plugs that were placed aseptically at the center of 2% MEA plates. Three replicates of each culture were incubated at 10–35 C at 5 degree intervals. Measurements of the diameters of the cultures were taken after 7 and 14 d and averages were computed.

RESULTS

DNA sequence analyses.—Three sets of sequence data were analysed. LSU data (FIG. 1) were used to determine the position of species at the genus level. Based on the results of the LSU data (FIG. 1) ITS and β-tubulin sequences of known *Ophiostoma* and *Sporothrix* species related to the species in this study were selected to resolve species level relationships (FIGS. 2, 3). In all species of the *O. stenoceras-S. schenckii* complex (dotted line box in FIG. 1) the β-tubulin gene contains intron 5 but not intron 4 while almost all known *Ophiostoma* spp. outside the complex contain intron 4 but not intron 5 (Zipfel et al 2006). It thus is impossible to meaningfully align the β-tubulin sequences of members of these two discordant groups. The intron differences restricted the choice of species included in the β-tubulin dataset (FIG. 3) and consequently the ITS dataset (FIG. 2). ITS and β-tubulin sequences of *S. schenckii* obtained from GenBank originated from different studies and isolates, making it impossible to combine ITS and β-tubulin datasets.

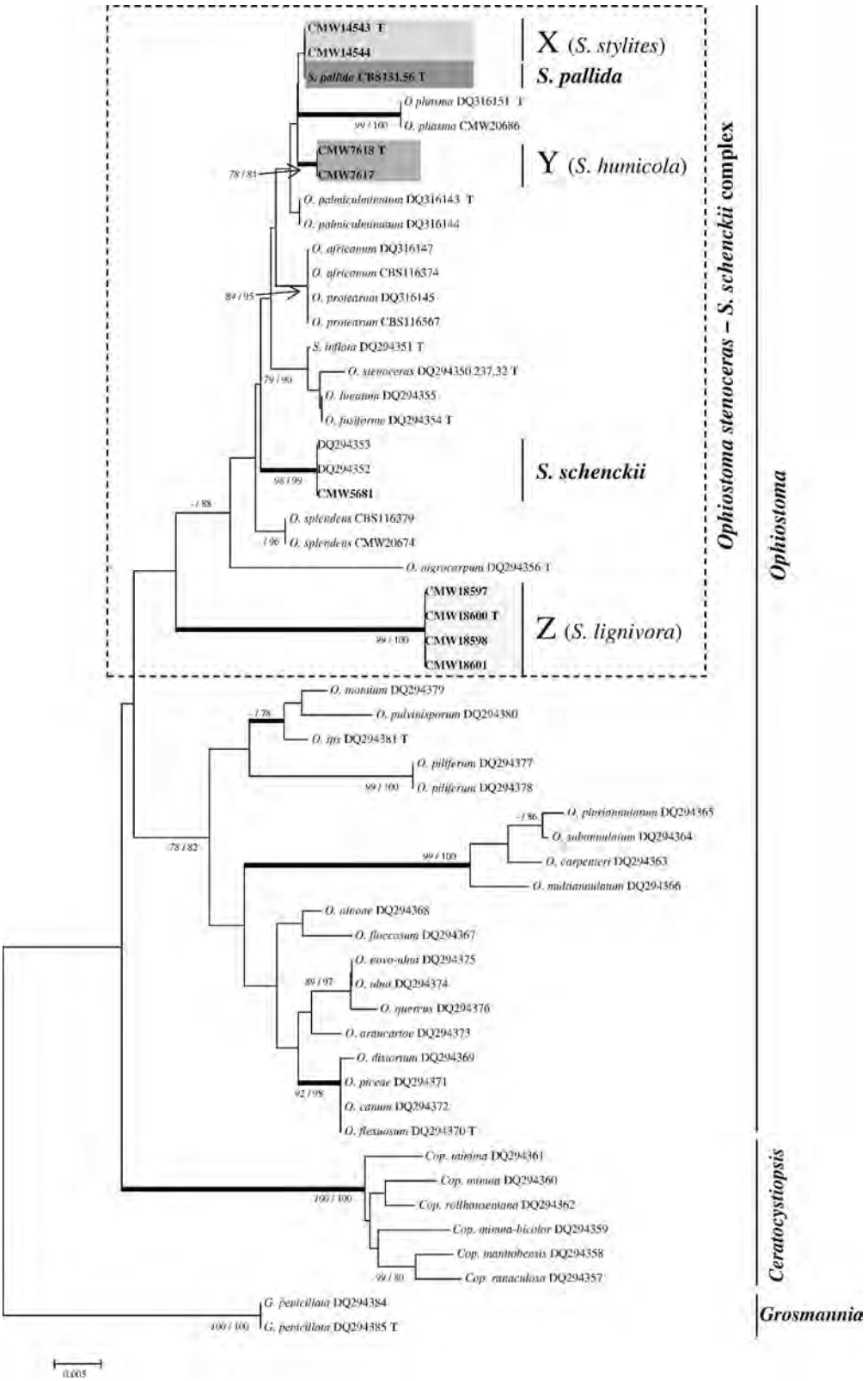

FIG. 1. Neighbor-joining tree based on LSU sequence data, demonstrating the location of the *O. stenoceras-S. schenckii* complex in the *Ophiostomatales*. Isolate numbers of sequences obtained during this study are printed in bold type, and the different species are presented in different shades of gray. The tree is rooted with *Grosmannia* as outgroup. Bootstrap values (> 75%) for maximum parsimony (italics) and neighbor joining analyses (normal font) are given at the nodes. Branches with Bayesian support of > 95% printed in bold.

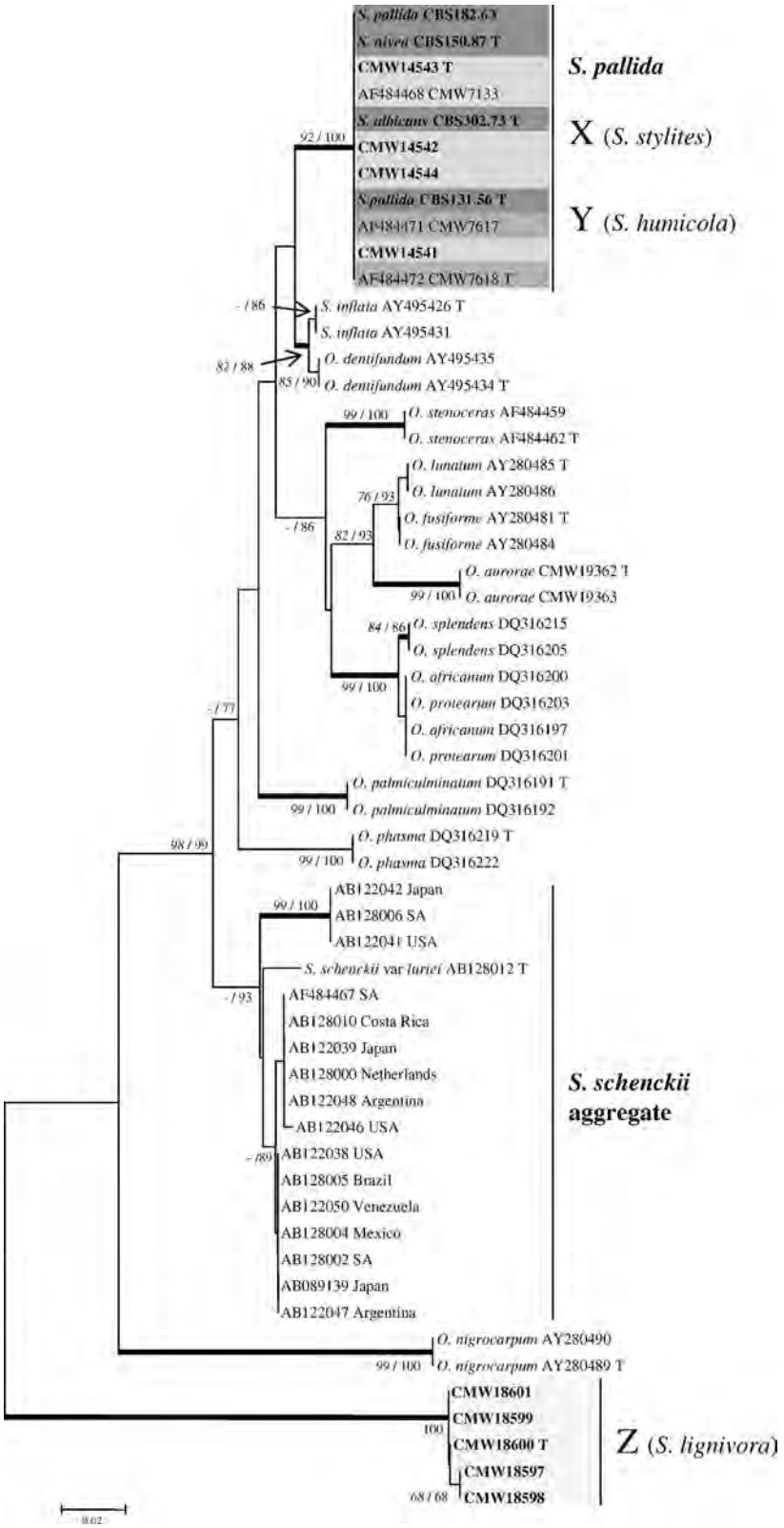

FIG. 2. Unrooted neighbor joining tree based on sequences of the ITS 1 and 2 regions, including the 5.8S gene, of selected isolates in the *O. stenoceras*-*S. schenckii* complex. Isolate numbers of sequences obtained during this study are printed in bold type, and the different species are presented in different shades of grey. Bootstrap values (> 75%) for maximum parsimony (italics) and neighbour joining (normal font) are given at branching points. Branches with Bayesian support of > 95% are printed in bold.

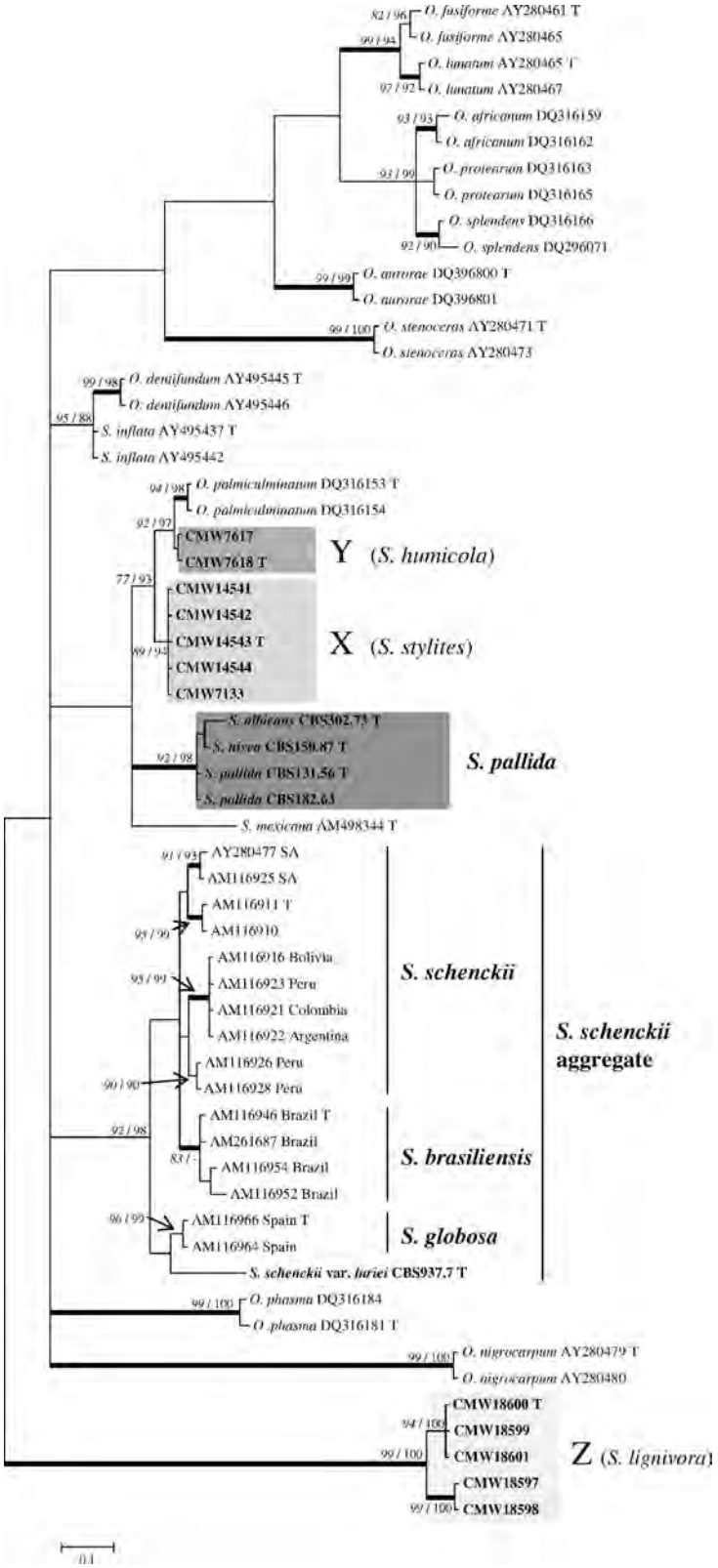

FIG. 3. Likelihood tree obtained from Bayesian analysis of the partial β-tubulin gene sequences (introns included) of selected isolates in the *O. stenoceras-S. schenckii* complex. Isolates obtained during this study are printed in boldface, and the species under investigation are presented in different shades of gray. Bootstrap values (> 75%) for maximum parsimony (italics) and neighbor joining (normal font) are given at the nodes. Branches with Bayesian support of > 95% are printed in bold.

The aligned sequences showed 691 parsimony-informative sites for the LSU sequences, 380 for ITS and 286 for β-tubulin. Maximum parsimony analysis of the LSU, ITS and β-tubulin sequences respectively resulted in a total of 72, 60 and 27 trees, with tree lengths of 253, 232 and 154. Consistency indices (CI) were = 0.546, 0.737 and 0.608, and retention indices (RI) were = 0.877, 0.949 and 0.896.

Based on LSU sequences the *Sporothrix* isolates from utility poles and soil formed three distinct groups (FIG. 1; X, Y and Z), all within the *O. stenoceras-S. schenckii* complex. The groups corresponded with geographic regions and hosts from which they were isolated. Group X represented isolates from pine poles in Plettenberg Bay; group Y contained isolates from soil, previously referred to as ''environmental'' *S. schenckii* isolates (de Beer et al 2003); and group Z were isolates from *Eucalyptus* poles from both St Lucia and Stellenbosch. The clinical isolates of *S. schenckii* formed a distinct group. LSU sequences of Group X isolates were identical to the sequence of the ex-type isolate of *S. pallida* (FIG. 1). This species thus formed a monophyletic lineage with Group X. Groups X and Y were most closely related to *O. phasma* Roets, Z.W. de Beer & M.J. Wingf. and *O. palmiculminatum* Roets, Z.W. de Beer & M.J. Wingf. but were distinct from both species. Group Z formed a distinct clade basal to all other species in the *O. stenoceras-S. schenckii* complex.

A GC-rich area of the ITS 1 region (approx positions 65–130) of several isolates in this study was difficult to sequence. In some cases complete ITS 1 sequences were obtained only after several sequencing attempts with primers ITS1-F, ITS2 and ITS4. However complete ITS 1 sequences were not obtained for five of the isolates (CMW14541, CBS182.63, CBS302.73, CBS150.87, CBS131.56). Based on ITS sequences (FIG. 2) isolates residing in X and Y grouped with *S. albicans*, *S. nivea* and *S. pallida*. Problems relating to sequencing the GC-rich area could have contributed to the lack of resolution in this group, to which *S. inflata* and *O. dentifundum* grouped most closely. Group Z was basal in the tree and clearly distinct from any of the other species. *Sporothrix schenckii* isolates formed a monophyletic group with relatively strong bootstrap support.

The tree derived from the β-tubulin data (FIG. 3) significantly improved resolution among closely related species. Isolates formed five distinct groups with significant statistical support. Group X emerged as distinct from the *S. pallida* group, which included the isolates accessioned in CBS as *S. albicans*, *S. nivea* and *S. pallida*, including the ex-type isolates of all three species. Isolates in the *S. pallida* group originated from Europe and Asia. Group Y isolates clustered most closely to *O. palmiculminatum*. Group Z grouped basal in the unrooted tree. As in the ITS tree the clinical isolates formed a well supported monophyletic lineage. Included were two lineages respectively representing the ex-type strains of *S. brasiliensis* Marimon, Gené, Cano & Guarro and *S. globosa* Marimon, Gené, Cano & Guarro. The ex-type strain of *S. schenckii* var. *luriei* Ajello & Kaplan grouped distinct from all the other species in the *S. schenckii* aggregate. The remaining isolates, all identified in the studies from which they originated as *S. schenckii* of human origin, were divided in four relatively well supported subgroups.

Morphology.—Teleomorph structures were not observed for any of the isolates. Colonies representing groups X (FIG. 4a) and Y (FIG. 4d) had a flattened morphology with a smooth texture, similar to those of *S. pallida* (FIG. 4m) and *S. schenckii* (FIG. 4j). The clinical *S. schenckii* isolates darkened with age while those representing groups X and Y, as well as members of the *S. pallida* cluster, remained white to cream colored. Conidia of isolates residing in groups X and Y and those of the *S. pallida* cluster were similar in shape (FIG. 4b, e, o). Denticles of those in Group Y (FIG. 4f) appeared to be more prominent (in this case more distinctly subcylindrical) than those of either Group X or *S. pallida* (FIG. 4n). Denticles of all three groups were formed on both terminal and intercalary conidiogenous cells.

The colony appearance and conidial morphology of Group Z isolates differed distinctly from those of the other isolates examined. The light olivaceous Group Z cultures had prominent aerial mycelium yielding a fluffy texture. The isolates from St Lucia (CMW18597, CMW18598) were slightly darker than those from Stellenbosch (CMW18599, CMW18600, CMW18601). The conidia of all Group Z isolates varied from oval to oblong, with longer conidia becoming medially constricted (FIG. 4h, i).

All isolates from utility poles (groups X and Z) and soil (Group Y) grew faster than the clinical isolates at all temperatures tested (FIG. 5). Group X isolates attained an average 37 mm diam at 25 C in 14 d in the dark while the clinical *S. schenckii* isolates grew to 20 mm diam. *Sporothrix pallida* showed the same growth rate (40 mm) at 25 C as Group X isolates but grew more slowly at 30 C and 35 C. All Group X isolates grew at this temperature. *Sporothrix pallida* grew faster at 10 C than did members of X, Y and Z. Group Y isolates attained 40 mm diam at 25 C in 14 d, while Z isolates reached 50 mm diam in the same time and under the same conditions.

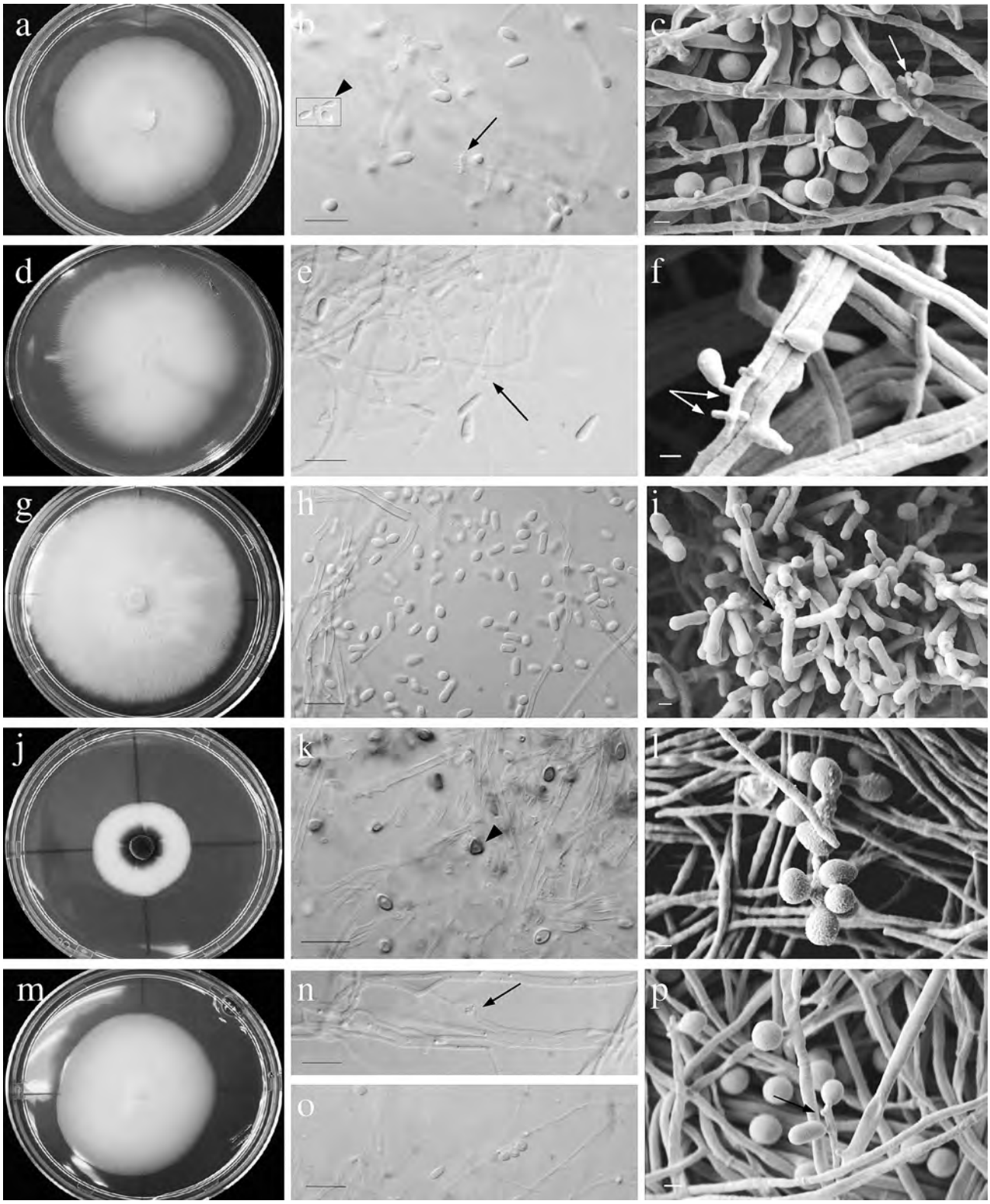

FIG. 4. a. *Sporothrix stylites* culture (CBS 118848). b. conidiogenous cell with denticles, conidia and secondary conidium (arrow head), c. denticles and conidia, d. *Sporothrix humicola* culture (CBS 118129), e. denticles and conidia, f. conidia and denticles; g. *Sporothrix lignivora* culture (CBS 119148), h. various conidial shapes, i. conidia and denticles; j. *Sporothrix schenckii* culture (CMW 7615), k. dark pigmented triangular conidia (arrow head), l. conidia; m. *Sporothrix pallida* culture (CBS 131.56), n, o. denticles and conidia, p. denticles and conidia. (Arrows indicate denticles). Black bar = 10 µm, white bar = 1 µm.

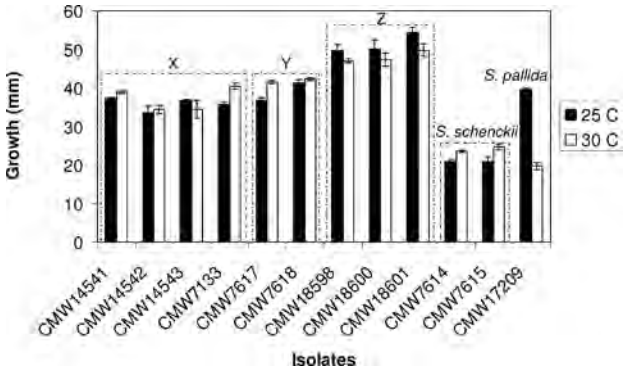

FIG. 5. Average growth of isolates of selected *Sporothrix* species in millimeters at 25 C and 30 C after 14 d on MEA. Species included were *Sporothrix stylites* (X), *S. humicola* (Y), *S. lignivora* (Z), *S. schenckii* and *S. pallida*.

TAXONOMY

Based on the analyses of the LSU, ITS and β-tubulin sequence data, as well as morphological and cultural differences, results of this study lead us to recognize isolates residing in groups X, Y and Z as distinct taxa. They consequently are described here as *S. stylites*, *S. humicola* and *S. lignivora* respectively. *Sporothrix pallida*, *S. albicans* and *S. nivea* are similar based on phylogenetic and morphological descriptions. The last two species therefore are synonymized with *S. pallida*, the first of the three species to be described.

Sporothrix stylites de Meyer, Z.W. de Beer & M.J. Wingf., sp. nov. FIG. 4a–c

Etymology. Stylites or ''pillar saints'' were ascetics in the 4th century, who spent their days fasting and praying on tops of pillars (*stylos* in Greek) or poles, a habitat similar to that from which this fungus was isolated.

Coloniae stramineae, laeves, interdum funiculosae, compactae, planae vel sulcatae cerebro similes. Hyphae 1–2 μm latae. Conidiophorae micro- vel semimacronematae, solitaeiae, rectae, simplices, non septatae, hyalinae. Cellulae conidiogenae polyblasticae, subcontractae, micronematae, hyalinae, septatae, orthotropice e hyphis non modificatis orientes, parte apicali cum denticulis maculiformibus vel subcylindricis 0.5 × 1 μm. Conidia (2–)2.5–4(–5.5) × (1–)1.5–2(–2.5) μm, proxime in denticulis orientia, hyalina, non septata, laevia, guttiformia vel fusiformia, basi acuminata, interdum cum conidiis secondariis. Coloniae ca. 37 mm diam post 14 d in 2% MEA ad 25 C in tenebris. Crescunt optime 25–30 C.

Cultures more straw than buff, usually smooth, sometimes becoming funiculose, compact, flat or furrowed brain-like (FIG. 4a). *Hyphae* 1–2 μm wide. *Conidiophores* micro- to semimacronematous, solitary, straight, simple, nonseptate, hyaline, conidiophore reduced to conidiogenous cell. *Conidiogenous cells* (FIG. 4b) polyblastic, discrete, slightly tapering, micronematous, hyaline, septate, arising orthrotropically from undifferentiated hyphae, apical part bearing denticles. *Denticles* (FIG. 4b) blotch-like to subcylindrical 0.5 × 1 μm. *Conidia* (FIG. 4c) (2–)2.5–4(–5.5) × (1–)1.5–2(–2.5) μm, produced directly on denticles, hyaline, nonseptate, smooth- and thin-walled, guttiliform to fusiform with a pointed base, secondary conidia sometimes formed (FIG. 4b, insert). Isolates attaining average 37 mm diam in 14 d on 2% MEA at 25 C in the dark. Optimum growth 25–30 C.

Specimens examined. SOUTH AFRICA. WESTERN CAPE: Plettenberg Bay. Isolated from pine pole at soil level, Jun 2003, E.M. de Meyer (HOLOTYPE, PREM 59278; culture ex-holotype, CMW 14543 = CBS 118848). Same location, date and collector (PREM 59276, culture CMW 14541 = CBS 115868; PREM 59277, culture CMW 14542 = CBS 115872; PREM 59279, culture CMW 14544 = CBS 115869). SOUTH AFRICA. FREE STATE: Bloemfontein. Isolated from *Rosa* sp., Z.W. de Beer (CMW 7133).

The species most closely related to *S. stylites* are *O. phasma* and *O. palmiculminatum*. Unlike these two species *S. stylites* is not known to form teleomorphic structures. *Ophiostoma palmiculminatum* is similar to *S. stylites* in culture. In shape conidia of *S. stylites* are similar to but smaller than those of *O. palmiculminatum*. *Ophiostoma phasma* has two distinct conidial forms. One of these is similar to but larger than conidia of *S. stylites*, while conidia of the other type are globose and very different from those of the new species. *Sporothrix schenckii* differs from *S. stylites* in that the cultures of the latter species do not darken with age (FIG. 4j). This phenomenon results from the formation in *S. schenckii* of dark secondary conidia (Mackinnon et al 1969) (FIG. 4k), structures that are absent in *S. stylites*. *Sporothrix schenckii* also grows significantly more slowly than *S. stylites* at all temperatures. *Sporothrix stylites* is similar to *S. pallida* in culture and micromorphology.

Sporothrix humicola de Meyer, Z.W. de Beer & M.J. Wingf., sp. nov. FIG. 4d–f

Etymology. This name refers to the soil substrate from which this species was isolated.

Coloniae stramineae, laeves, interdum funiculosae, lanuginosae. Hyphae 1–1.5 μm latae. Conidiophorae micro- vel semimacronematae, solitariae, rectae, simplices, non septatae, hyalinae. Cellulae conidiogenae polyblasticae, subcontractae, micronematae, hyalinae, septatae, orthotropice e hyphis non modificatis orientes, parte apicali cum denticulis maculiformibus vel subcylindricis 0.5 × 1 μm, in hyphis vegetativis vel parte apicali incrassata factis. Conidia 5–8(–14) × 1.5–2.5 μm, proxime in denticulis orientia, hyalina, non septata, laevia, guttiformia vel fusiformia, basi acumi-

nata. Coloniae ca. 40 mm diam post 14 d in 2% MEA ad 25 C in tenebris. Crescunt optime ad 30 C.

Cultures more straw than buff, usually smooth, sometimes becoming funiculose and woolly (Fig. 4d). *Hyphae* 1–1.5 µm wide. *Conidiophores* micro- to semimacronematous, solitary, straight, simple, nonseptate, hyaline, conidiophore reduced to conidiogenous cell. *Conidiogenous cells* (Fig. 4e) polyblastic, discrete, slightly tapering, micronematous, hyaline, septate, arising orthrotropically from undifferentiated hyphae, apical part bearing denticles. *Denticles* (Fig. 4f) blotch-like to subcylindrical 0.5 × 1 µm, produced on vegetative hyphae or thickened apical part. *Conidia* (Fig. 4f) 5–8(–14) × 1.5–2.5 µm produced directly on denticles, hyaline, nonseptate, smooth- and thin-walled, guttiliform to fusiform with pointed base. Isolates attaining an average 40 mm diam in 14 d on 2% MEA at 25 C in the dark. Optimum growth at 30 C.

Specimens examined. SOUTH AFRICA. GAUTENG. Isolated from soil, H.F. Vismer (HOLOTYPE, PREM 59282; culture ex-holotype CMW 7618 = CBS 118129). Same location, date and collector (PREM 59281, culture CMW 7617 = CBS 1120256).

Sporothrix humicola is morphologically similar to *S. stylites* and *S. pallida*, as well as to the anamorphs of *O. phasma* and *O. palmiculminatum*, although it has substantially larger conidia than *O. phasma* and *O. palmiculminatum*. Conidial size does not reliably distinguish *S. humicola* from the *S. pallida* group. As with *S. stylites*, *S. humicola* is similar to *S. schenckii* but the cultures and conidia do not darken with age as do *S. schenckii* and *S. mexicana* Marimon, Gené, Cano & Guarro.

Sporothrix lignivora de Meyer, Z.W. de Beer & M.J. Wingf., sp. nov. Fig. 4g–i

Etymology. This name refers to the wood destroying capability of the fungus.

Coloniae olivaceae viridescentes, subprominentes, lanuginosae, nonnullis cum hyphis aeriis. Hyphae (0.5–)1–2(–3.5) µm latae. Conidiophorae micro- vel semimacronematae, ad cellulas conidiogenas deminutae, mononematae, nonseptatae, determinatae, solitariae, rectae, simplices, hyalinae. Cellulae conidiogenae polyblasticae subcontractae, discretae, terminales, parte apicali cum denticulis inconspicuis. Conidia (2–)2.5–4(–4.5) × (1–)1.5–2 µm, solitaria, hyalina, non septata, laeves, forma magnitudineque diversa, oblonga, in centro subconstricta, basi manifeste truncata. Coloniae ca. 50 mm diam post 14 d in 2% MEA ad 25 C in tenebris. Crescunt optime ad 25 C, etiam bene ad 35 C, sed haudquaquam ad 10 C.

Cultures are greenish olivaceous (23'''b), slightly raised with a woolly texture (Fig. 4g). Some aerial hyphae are present. *Hyphae* (0.5–)1–2(–3.5) µm wide. *Conidiophores* micro- to semimacronematous, conidiophores reduced to conidiogenous cells, mononematous, nonseptate, determinate, solitary, straight, simple, hyaline. *Conidiogenous cells* (Fig. 4h) polyblastic, slightly tapering, discrete, terminal, apical part bearing inconspicuous denticles. *Conidia* (Fig. 4i) (2–)2.5–4(–4.5) × (1–)1.5–2 µm, solitary, hyaline, nonseptate, smooth walled, diverse in shape and size, oblong, slightly constricted at center. Distinctly truncate base. Isolates attaining an average 50 mm diam in 14 d on 2% MEA at 25 C in the dark. Optimum growth at 25 C, with good growth at 35 C but no growth at 10 C.

Specimens examined. SOUTH AFRICA. WESTERN CAPE: Stellenbosch. Isolated from eucalypt pole at soil level, Oct 2003, E.M. de Meyer (HOLOTYPE, PREM 59284, culture ex-holotype CMW 18600 = CBS 119148). Same location, date and collector (PREM 59283, culture CMW18601 = CBS 119149; PREM 59285, culture CMW 18599 = CBS 119147). SOUTH AFRICA. KWAZULU-NATAL: St Lucia. Isolated from eucalypt pole at soil level, May 2003, E.M. de Meyer (PREM 59286, culture CMW18598 = CBS 119146; PREM 59287, culture CMW 18597).

Sporothrix lignivora is distinguished by the shape and size of its conidia. No other *Sporothrix* species or *Ophiostoma* with *Sporothrix* anamorphs has these distinctive conidia. The most closely related species phylogenetically is *Ophiostoma nigrocarpum* (R.W. Davidson) de Hoog. This is a species associated with conifer-infesting bark beetles, which constitute a specific niche very different from that occupied by *S. lignivora*. *S. lignivora* differs in morphology from the anamorph of *O. nigrocarpum*, which has small (3–5 × 1.5–3 µm) ovoid conidia and is gray in culture.

Morphological descriptions of *S. pallida*, *S. albicans* and *S. nivea* as well as examination of cultural and microscopic characters shows no significant differences between these species. Growth rate at different temperatures also yielded similar results (data not shown). DNA sequence data of LSU, ITS and β-tubulin regions support the synonymy of *S. albicans* and *S. nivea* with *S. pallida*.

Sporothrix pallida (Tubaki) Matsush. Icones microfungorum a Matsushima lectorum (Kobe): 143 (1975)
≡ *Calcarisporium pallidum* Tubaki, *Nagaoa* 5:13 (1955)
= *Sporothrix albicans* S.B. Saksena, *Current Science* 34:318 (1965)
= *Sporothrix nivea* Kreisel & Schaver, *Journal of Basic Microbiology* 25:654 (1985)

DISCUSSION

On the basis of phylogenetic and morphological data, three new *Sporothrix* species from South Africa that resemble the human pathogen *S. schenckii* are

recognized. Isolates from pine poles and rosebush wood have been described as *S. stylites* and those from eucalypt poles as *S. lignivora*. Isolates from soil that previously were referred to as ''environmental'' isolates of *S. schenckii* (de Beer et al 2003) have been described as *S. humicola*.

Sporothrix stylites were isolated in the Western Cape Province from pine poles. The closely related *O. palmiculminatum* also originate from this region. The latter species however are best known based on their sexual state and they are specifically found in the infructescences of certain *Protea* spp. that occur only as native species in this area (Roets et al 2006a). The similar geographic origin of the fungi in question might be coincidental, but it also could indicate a common origin or association in relatively distant evolutionary history.

On the basis of phylogenetic analyses *S. stylites* and *S. humicola* also are related closely to *S. mexicana*, the recently described species from Mexico (Marimon et al 2007). Although *S. mexicana* also came from soil the morphology of the hyaline conidia of *S. humicola* clearly differs from the brown, thick-walled conidia produced by *S. mexicana*.

The third species grouping with *S. stylites* and *S. humicola*, namely *S. pallida*, has been a major source of historical confusion with *S. schenckii*. Closely clustered with *S. pallida* in β-tubulin sequence analysis were the ex-type isolates of two other seldom-used *Sporothrix* names, *S. albicans* and *S. nivea*. These isolates all show a pattern of isolation suggestive of saprotrophy; *S. pallida* is known from *Stemonitis* slime mold infructescences and from soil, while *S. albicans* was based on a soil isolate (Saksena 1965) and *S. nivea* on isolations from activated sludge in a sewage treatment facility (Kreisel and Schauer 1985). Based on ITS sequences alone members of the *S. pallida* cluster could not be distinguished from *S. stylites* and *S. humicola*. However β-tubulin data separated *S. stylites* and *S. humicola* from the Eurasian isolates. The Eurasian isolates formed a strongly supported monophyletic group, with only a few bp differences in β-tubulin distinguishing them. The first of these species to be described was *S. pallida*, as *Calcarisporium pallidum* Tub. (Tubaki 1955). Morphological examination supports the published descriptions in showing no important differences among these isolates, and tests for growth rates at different temperatures also show highly uniform results (data not shown). We consider the names *S. albicans* and *S. nivea* to be synonyms of *S. pallida*.

De Hoog (1974) listed both *S. albicans* and *Calcarisporium pallidum* Tubaki (= *S. pallida*) as synonyms of *S. schenckii* and also included the anamorphs of *O. stenoceras* in the same species concept. Matsushima (1975) however treated *C. pallidum* as distinct from *S. schenckii* and introduced the new combination, *S. pallida* (Tubaki) Matsushima. He claimed to have collected a new isolate from a burdock (*Arctium lappa*) seed. Although we have not attempted to obtain this material his illustrations and description, as well as the source of isolation, are compatible with the confirmed isolates of the molecularly reconceived *S. pallida*.

In an attempt to determine the extent to which *S. pallida* isolates accounted for the *S. schenckii*-like nonpathogens isolated in epidemiological studies such as those of Cooper et al (1992), we contacted various researchers involved in these outbreaks and attempted to obtain isolates. Databases of all known fungal culture collections also were searched for cultures. It would appear however that only confirmed pathogenic *S. schenckii* isolates were ever retained in these studies, most notably in the investigation of the dramatic 1988 sporotrichosis epidemic (Dixon et al 1991) that produced 84 known cases in 15 American states. Eight of the environmental *S. schenckii*-like isolates connected to this outbreak, all of which were from contaminated *Sphagnum* peat moss, clearly comprised *S. schenckii* s. str., the only pathogen in the material as seen in animal studies (Dixon et al 1992). The same isolation attempts however also yielded *O. stenoceras* as well as four additional morphogroups of *S. schenckii*-like isolates (Dixon et al 1991). One of these groups was composed of isolates expressing brown soluble pigment in culture, a feature incompatible with any of the species considered in the present study. The other three groups (II, V and VI) were pallid *S. schenckii*-like isolates not forming a teleomorph in culture and thus were compatible with *S. pallida* or *S. stylites* (Dixon et al 1991, Cooper et al 1992). They encompassed five distinct *Hae*III and *Msp*I restriction fragment types (Cooper et al 1992). Based on the results of the present study β-tubulin sequence analysis is strongly recommended in all future isolates obtained in such investigations. This complex is highly likely to contain additional undescribed species.

Sporothrix lignivora, isolated from eucalypt poles in the Western Cape and KwaZulu-Natal provinces, were distinct from *S. stylites* and *S. humicola* based on morphology and DNA sequence comparisons. Isolates of *Sporothrix lignivora* formed two subgroups based on β-tubulin sequences, and these groups corresponded with the geographic origin of the isolates. The isolates from KwaZulu-Natal were slightly darker in culture than those from the Western Cape. However differences based on DNA were too small to separate the two groups as distinct species.

Isolates of human origin formed a group distinct from the other *Sporothrix* and *Ophiostoma* species in this study based on LSU, ITS and β-tubulin sequences. Our data confirmed the views of de Beer et al (2003) suggesting that at present no *Ophiostoma* species can be linked to *S. schenckii*. Our ITS dataset included *S. schenckii* sequences of various origins from the studies of de Beer et al (2003) and Watanabe et al (2004). Sequences from the studies of Marimon et al (2006, 2007) were included in our β-tubulin analyses, and our results showed that all clinical isolates form a monophyletic lineage consisting of several species within the *Ophiostomatales*. However because different gene regions and isolates were used in the various studies the groups in the ITS tree could not be matched with those in the β-tubulin tree that included the species described by Marimon et al (2007). To resolve the species boundaries in the *S. schenckii* aggregate a multigene study of this group is necessary; this study will include isolates from all the subgroups and cover all gene regions of which sequence data have been published.

Species in the *O. stenoceras-S. schenckii* complex originate from a wide variety of niches and substrates. These include associations with infructescences of *Protea* spp. (*O. phasma*, *O. protearum*, *O. africanum*, *O. splendens*, *O. palmiculminatum*), deciduous trees (*O. lunatum*, *O. fusiforme*, *O. dentifundum*, *S. lignivora*), conifer-infesting bark beetles (*O. nigrocarpum*, *O. aurorae*) and one with conifer wood (*S. stylites*). The fungi also can be found in soil, peat moss, plants of various kinds and human clinical materials. Two species in the *O. stenoceras-S. schenckii* complex that were not included in the study were *O. narcissi* Limber and *O. abietinum* Marm. & Butin (de Beer et al 2003). They come respectively from flower bulbs and conifer bark beetles. These niches do not seem to be ecologically related, and they do not correspond to any obvious pattern of association. However it is relevant to consider that the complex includes six species (*Sporothrix inflata*, *S. humicola*, *S. pallida*, *S. mexicana*, *S. schenckii* and *O. stenoceras*) that have been isolated directly from soil. *Ophiostoma narcissi* originates from flower bulbs growing in soil, and *S. stylites* and *S. lignivora* were isolated at ground level from poles secured in the soil. *S. schenckii* and *S. schenckii* var. *luriei* are most commonly isolated from human tissue, but soil and many types of vegetative material have been shown to be sources of confirmed pathogenic *S. schenckii* (Dixon et al 1992, Summerbell et al 1993, Vismer and Hull 1997, Ishizaki et al 2002). *Ophiostoma aurorae* originates from *Hylastes angustatus*, a root-infesting bark beetle that bores through the soil to reach the roots (Zhou et al 2006). The *Protea*-associated species are associated with insects, and at least three of them (*O. phasma*, *O. palmiculminatum*, *O. splendens*) appear to be vectored by mites (Roets et al 2006b). To date none of the *Protea*-associated species has been recorded from soil, but frequent contact between mites and soil is possible. This implies that in total 16 out of 24 species in the *O. stenoceras-S. schenckii* complex are from the soil or associated niches. Of the remaining six species, four (*O. lunatum*, *O. fusiforme*, *O. dentifundum*, *O. nigrocarpum*) are from wood and might be associated with bark beetles. These also might have occasional beetle-mediated contact with soil.

Genus *Ophiostoma* and its related genera *Ceratocystiopsis* and *Grosmannia* long have been considered northern hemisphere fungi with close bark beetle associations (Upadhyay 1981). However, of the 24 species placed in the *O. stenoceras-S. schenckii* complex based on phylogenetic data, 14 species have been recorded from South Africa and 10 of these have been found exclusively in South Africa. The discovery of *Ophiostoma* species associated with endemic *Protea* species in southern Africa (Wingfield et al 1988, Marais and Wingfield 2001, Roets et al 2006a) and the high biodiversity of *Ophiostoma* species from the *O. stenoceras-S. schenckii* complex in southern Africa raise many questions. For example it would be interesting to know whether *Ophiostoma* and its related genera originated in the soil and whether they might have a Gondwanaland-based origin. How the human pathogen *S. schenckii* originated is also an intriguing question, especially because the answer could have medical implications. These questions deserve consideration; answers are likely to emerge from more extensive sampling of these fungi in new environments.

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LITERATURE CITED

Aghayeva DN, Wingfield MJ, de Beer ZW, Kirisits T. 2004. Two new *Ophiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan. Mycologia 96:866–878.

———, ———, Kirisits T, Wingfield BD. 2005. *Ophiostoma dentifundum* sp. nov. from oak in Europe, characterized using molecular phylogenetic data and morphology. Mycol Res 109:1127–1136.

Arenas R, Miller D, Campos-Macias P. 2007. Epidemiological data and molecular characterization (mtDNA) of *Sporothrix schenckii* in 13 cases from Mexico. Int J Dermatol 46:177–179.

Berbee ML, Taylor JW. 1992. 18S ribosomal RNA gene sequence characters place the human pathogen *Sporothrix schenckii* in the genus *Ophiostoma*. Exp Mycol 16:87–91.

Brown R, Weintroub D, Simpson MW. 1947. Timber as a source of sporotrichosis infection. In: Sporotrichosis infection on mines of the Witwatersrand. Proceedings of the Transvaal Mine Medical Officers' Association, Dec 1944. Johannesburg, South Africa: The Transvaal Chamber of Mines. p 5–33.

Callens SF, Kitetele F, Lukun P, Lelo P, van Rie A, Behets F, Colebunders R. 2006. Pulmonary *Sporothrix schenckii* infection in a HIV positive child. J Trop Pediatr 52:144–146.

Cooper CR Jr, Breslin BJ, Dixon DM, Salkin IF. 1992. DNA typing of isolates associated with the 1988 sporotrichosis epidemic. J Clin Microbiol 30:1631–1635.

de Beer ZW, Harrington TC, Vismer H, Wingfield BD, Wingfield MJ. 2003. Phylogeny of the *Ophiostoma stenoceras-Sporothrix schenckii* complex. Mycologia 95: 434–441.

de Hoog GS. 1974. The genera *Blastobotrys*, *Sporothrix*, *Calcarisporium* and *Calcarisporiella* gen. nov. Stud Mycol 7:1–84.

———. 1993. *Sporothrix*-like anamorphs of *Ophiostoma* species and other fungi. In: Wingfield MJ, Seifert KA, Webber JF, eds. *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. Minnesota: APS Press. p 53–60.

de Meyer EM. 2007. Fungi from utility poles in South Africa: Taxonomy, phylogeny and effect on wood (Master's thesis). Pretoria, South Africa: Univ Pretoria. 139 p.

Dixon DM, Duncan RA, Hurd NJ. 1992. Use of a mouse model to evaluate clinical and environmental isolates of *Sporothrix* species from the largest U.S. epidemic of sporotrichosis. J Clin Microbiol 30:951–954.

———, Salkin IF, Duncan RA, Hurd NJ, Haines J, Kemna ME, Coles B. 1991. Isolation and characterization of *Sporothrix schenckii* from clinical and environmental sources associated with the largest U.S. epidemic of sporotrichosis. J Clin Microbiol 29:1106–1113.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol 61:1323–1330.

Hardman S, Stephenson I, Jenkins DR, Wiselka MJ, Johnson EM. 2005. Disseminated *Sporothrix schenckii* in a patient with AIDS. J Infect 51:e73–e77.

Heller HM, Fuhrer J. 1991. Disseminated sporotrichosis in patients with AIDS: case report and review of the literature. AIDS 5:1243–1246.

Helm MAF, Berman C. 1947. The clinical, therapeutic and epidemiological features of the sporotrichosis infection on the mines. In: Sporotrichosis infection on mines of the Witwatersrand. Proceedings of the Transvaal Mine Medical Officers' Association, Dec 1944. Johannesburg, South Africa: The Transvaal Chamber of Mines. p 59–67.

Howard DH, Orr FG. 1963. Comparison of strains of *Sporotrichum schenckii* isolated from nature. J Bacteriol 85:816–821.

Ishizaki H, Kawasaki M, Mochizuki T, Jin X-Z, Kagawa S. 2002. Environmental isolates of *Sporothrix schenckii* in China. J Med Mycol 43:257–260.

———, ———, Aoki M, Miyaji M, Nishimura K, Garcia Fernandez JA. 1996. Mitochondrial DNA analysis of *Sporothrix schenckii* in Costa Rica. J Med Vet Mycol 34: 71–73.

———, ———, ———, Matsumoto T, Padhye AA, Mendoza M, Negroni R. 1998. Mitochondrial DNA analysis of *Sporothrix schenckii* in North and South America. Mycopathologia 142:115–118.

———, ———, ———, Vismer HF, Muir DCG. 2000. Mitochondrial DNA analysis of *Sporothrix schenckii* in South Africa and Australia. Med Mycol 38:433–436.

Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. NAR 30:3059–3066.

Kirisits T. 2004. Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi. In: Lieutier F, Day KR, Battisti A, Grégoire JC, Evans HF, eds. Bark and wood boring insects in living trees in Europe, a synthesis. Dordrecht, The Netherlands: Kluwer Academic Press. p 181–235.

Kreisel H, Schauer F. 1985. *Sporothrix nivea* n. sp.—ein Methanol und Kohlenwasserstoffe verwertender Pilz. J Basic Microbiol 25:653–661.

Kumar S, Tamura K, Nei M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163.

Lin J, Kawasaki M, Aoki M, Ishizaki H, You G, Li R. 1999. Mitochondrial DNA analysis of *Sporothrix schenckii* clinical isolates from China. Mycopathologia 148:69–72.

Mackinnon JE, Conti Diaz IA, Gezuele E, Civila E, Da Luz S. 1969. Isolation of *Sporothrix schenckii* from nature and considerations on its pathogenicity and ecology. Sabouraudia 7:38–45.

Marais GJ, Wingfield MJ. 2001. *Ophiostoma africanum* sp. nov., and a key to ophiostomatoid species from *Protea* infructescences. Mycol Res 105:240–246.

Mariat F. 1975. Observations sur l'ecologie de *Sporothrix schenckii* et de *Ceratocystis stenoceras* en Corse et en Alsace, provinces françaises indemnés de sporotrichose. Sabouraudia 13:217–225.

Marimon R, Gene J, Cano J, Trilles L, Dos Santos Lazera M,

Guarro J. 2006. Molecular phylogeny of *Sporothrix schenckii*. J Clin Microbiol 44:3251–3256.

———, Cano J, Gene J, Sutton DA, Kawasaki M, Guarro J. 2007. *Sporothrix brasiliensis*, *S. globosa*, and *S. mexicana*, three new *Sporothrix* species of clinical interest. J Clin Microbiol 45:3198–3206.

Matsushima T. 1975. Icones microfungorum a Matsushima lectorum. Kobe, Japan: Publ by the author. 209 p, 415 pl.

Mora-Cabrera M, Alonso RA, Ulloa-Arvizu R, Torres-Guerrero H. 2001. Analysis of restriction profiles of mitochondrial DNA from *Sporothrix schenckii*. Med Mycol 39:439–444.

Neyra E, Fonteyne P-A, Swinne D, Fauche F, Bustamante B, Nolard N. 2005. Epidemiology of human sporotrichosis investigated by amplified fragment length polymorphism. J Clin Microbiol 43:1348–1352.

O'Reilly LC, Altman SA. 2006. Macrorestriction analysis of clinical and environmental isolates of *Sporothrix schenckii*. J Clin Microbiol 44:2547–2552.

Pijiper A, Pullinger BD. 1927. An outbreak of sporotrichosis among South African native miners. Lancet 2:914–915.

Rayner RW. 1970. A mycological colour chart. Kew, UK: CMI.

Riddell RW. 1950. Permanent stained mycological preparations obtained by slide culture. Mycologia 42:265–270.

Rocha MM, Dassin T, Lira R, Lima EL, Severo LC, Londero AT. 2001. Sporotrichosis in patient with AIDS: report of a case and review. Rev Iberoam Micol 18:133–136.

Roets F, Crous PW, Wingfield MJ, de Beer ZW, Dreyer LL. 2006b. Discovery of fungus-mite-mutualism within a unique niche of the Cape Floral Kingdom. [Doctoral thesis]. Stellenbosch, South Africa: Univ Stellenbosch.

———, de Beer ZW, Dreyer LL, Crous PW, Zipfel R, Wingfield MJ. 2006a. Multigene phylogeny of *Ophiostoma* spp. associated with *Protea* infructescences including two new species. Stud Mycol 55:203–216.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.

Saksena SB. 1965. A new species of *Sporothrix*. Curr Sci 34: 318.

Seifert KA. 1993. Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In: Wingfield MJ, Seifert KA, Webber JF, eds. *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. Minnesota: APS Press. p 141–151.

Shaw JC, Levinson W, Montanaro A. 1989. Sporotrichosis in the acquired immunodeficiency syndrome. J Am Acad Derm 21:1145–1147.

Six DL. 2003. Bark beetle-fungus symbioses. In: Bourtzis K, Miller TA, eds. Insect symbioses. New York: CRC Press. p 97–114.

Summerbell RC, Kane K, Krajden S, Duke EE. 1993. Medically important *Sporothrix* species and related Ophiostomatoid fungi. In: Wingfield MJ, Seifert KA, Webber JF, eds. *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. Minnesota: APS Press. p 185–192.

Suzuki K, Kawasaki M, Ishizaki H. 1988. Analysis of restriction profiles of mitochondrial DNA from *Sporothrix schenckii* and related fungi. Mycopathologia 103: 147–151.

Takeda Y, Kawasaki M, Ishizaki H. 1991. Phylogeny and molecular epidemiology of *Sporothrix schenckii* in Japan. Mycopathologia 116:9–14.

Travassos LR, Lloyd KO. 1980. *Sporothrix schenckii* and related species of *Ceratocystis*. Microbiol Rev 44:683–721.

Tubaki K. 1955. Studies on the Japanese Hyphomycetes (II). Fungicolous group. Nagaoa 5:11–40.

Upadhyay HP. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis*. Athens, Georgia: Univ of Georgia Press. 176 p.

Vismer HF, Hull PR. 1997. Prevalence, epidemiology and geographical distribution of *Sporothrix schenckii* infections in Gauteng, South Africa. Mycopathologia 137: 137–143.

Watanabe S, Kawasaki M, Mochizuki T, Ishizaki H. 2004. RFLP analysis of the internal transcribed spacer regions of *Sporothrix schenckii*. Jpn J Med Mycol 45:165–175.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis AM, Gelfand DH, Sninsky JJ, White TJ, eds. PCR protocols: a guide to methods and applications. San Diego: Academic Press. p 315–322.

Wingfield MJ, van Wyk PS, Marasas WFO. 1988. *Ceratocystiopsis proteae* sp. nov. with a new anamorph genus. Mycologia 80:23–30.

Zhou XD, de Beer ZW, Wingfield MJ. 2006. DNA sequence comparisons of *Ophiostoma* spp., including *Ophiostoma aurorae* sp. nov., associated with bark beetles in South Africa. Stud Mycol 55:273–281.

Zipfel RD, de Beer ZW, Jacobs K, Wingfield BD, Wingfield MJ. 2006. Multigene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. Stud Mycol 55:77–99.