Mycologia, 95(3), 2003, pp. 434–441.

Phylogeny of the *Ophiostoma stenoceras–Sporothrix schenckii* complex

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Abstract: *Ophiostoma stenoceras* is a well-known sapwood-colonizing fungus occurring on some coniferous and hardwood hosts in the Northern Hemisphere. In the Southern Hemisphere, the fungus has been reported only from New Zealand. The human pathogen, *Sporothrix schenckii*, has been suggested to be the anamorph of *O. stenoceras*. The aim of this study was to gain a better understanding of the phylogenetic relationship between these two species. The study also provided the opportunity to confirm the identity of some *Sporothrix* and *O. stenoceras*-like isolates recently collected from wood and soil around the world. For this purpose, the DNA sequence of internal transcribed spacer (ITS) regions of the ribosomal RNA operon was determined. Isolates of *O. nigrocarpum*, *O. albidum*, *O. abietinum*, *O. narcissi* and *O. ponderosae*, all morphologically similar to *O. stenoceras*, were included in the study. From phylogenetic analyses of the sequence data, four main clades were observed. These represented *O. stenoceras*, *O. nigrocarpum* and two separate groups containing isolates of *S. schenckii*. Our results confirm earlier suggestions that *S. schenckii* should be classified within the teleomorph genus *Ophiostoma* but support studies separating *O. stenoceras* and *S. schenckii*. *Ophiostoma albidum* and *O. ponderosae* should be considered synonyms of *O. stenoceras*. The status of *O. narcissi* and *O. abietinum* needs further clarification. The two groups within *S. schenckii* might represent two species, but this needs to be confirmed. This study represents the first reports of *O. stenoceras* from Colombia, Kenya, Uruguay and South Africa.

Key words: *abietinum*, *albidum*, ITS, *narcissi*, *nigrocarpum*, *ponderosae*, rDNA

INTRODUCTION

Ophiostoma stenoceras (Robak) Nannf. is a sapwood-colonizing fungus that first was described from ground wood pulp in Norway (Robak 1932). It since has been isolated from many other coniferous hosts, as well as some hardwood trees from the Northern Hemisphere (Davidson 1942, Griffin 1968, Otani 1988). In the Southern Hemisphere, *O. stenoceras* has been reported only from New Zealand (Farrell et al 1997, Schirp et al 1999). The fungus causes a slight gray stain on pine and spruce (Kåårik 1980) but is not considered economically important (Davidson 1942, Griffin 1968).

The first suggestion that *O. stenoceras* might represent the teleomorph of *Sporothrix schenckii* Hektoen & Perkins, the causative agent of human sporotrichosis, was made by Mariat (Mariat et al 1968, Mariat 1971a, b, Nicot and Mariat 1973). The relationship between *O. stenoceras* and *S. schenckii* since has been the subject of many research papers. A wide variety of taxonomic criteria were employed in these studies; they included conidium morphology, vitamin requirements, starch degradation, resistance to digestion by macrophage cells, immunological studies, cell wall components, neutral and polar lipid composition, carbohydrate composition, acid phosphatase isoenzyme patterns and pathogenicity studies (De Hoog 1974, Travassos and Lloyd 1980, Summerbell et al 1993). Molecular investigations included techniques such as DNA-DNA hybridisation, GC content (Mendonça-Hagler et al 1974) and mitochondrial restriction fragments (Suzuki et al 1988). The results of these studies often were contradictory, some suggesting that *S. schenckii* was the anamorph

Accepted for publication September 9, 2002.

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of *O. stenoceras* (Taylor 1970, De Hoog 1974) and others showing differences between the two species (Mendonça-Hagler et al 1974, Travassos et al 1974, Suzuki et al 1988). All these investigations were reviewed by Travassos and Lloyd (1980), as well as by Summerbell et al (1993). Although Travassos and Lloyd concluded that *S. schenckii* "bears little relation" to *O. stenoceras*, and a list of suggested criteria to distinguish between the two species was compiled (Summerbell et al 1993), the phylogenetic relationship between the two fungi never was clarified.

Berbee and Taylor (1992) confirmed with DNA sequencing that *S. schenckii* is phylogenetically related to *Ophiostoma*. The 18S rDNA gene sequenced in their study is highly conserved and does not exhibit sufficient variability to allow for distinction between closely related species. The aim of our study, therefore, was to gain a better understanding of the phylogenetic relationships between *O. stenoceras* and *S. schenckii*. To achieve this goal, we sequenced both the internal transcribed spacer (ITS) regions, including the 5.8S rRNA gene, of the ribosomal RNA operon. Isolates of *Ophiostoma* spp. that are morphologically similar to *O. stenoceras* also were included in the study. These were *O. nigrocarpum* (Davidson) De Hoog, *O. albidum* Mathiesen-Kåårik, *O. abietinum* Marmolejo & Butin, *O. narcissi* Limber and *O. ponderosae* (Hinds & Davidson) Hausner, Reid & Klassen. The study also provided us with the opportunity to confirm the identity of some *Sporothrix* and *O. stenoceras*-like isolates from wood and soil that recently have been collected from various Southern Hemisphere countries.

MATERIALS AND METHODS

Isolates.—Isolates resembling *O. stenoceras* and *O. nigrocarpum* (TABLE I) were collected from wood, bark beetles and soil from various countries, worldwide. Authenticated isolates of both these species, as well as one isolate each of *O. abietinum*, *O. albidum*, *O. narcissi* and *O. ponderosae*, were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, Netherlands, the American Type Culture Collection (ATCC), Manassas, Virginia, U.S.A., and the CABI Bioscience Genetic Resource Collection (IMI), Surrey, United Kingdom. The isolates of *O. abietinum* (C696) and *O. ponderosae* (C87) are associated with the types of the respective species, as is the *O. stenoceras* isolate CMW3202. The *O. albidum* isolate (C1190) was one of the isolates examined by Mathiesen-Kåårik (1953) when she described the species. Unfortunately no type material was designated for the species (Hunt 1956), and no culture representing type material exists.

Sporothrix schenckii isolates (TABLE I) were obtained from wood, soil and human patients. The rDNA sequence for the *S. schenckii* isolate from the USA (ATCC14284) was obtained from GenBank.

Where isolations were made from wood samples, these were initially incubated in Petri dishes with moist tissue paper at room temperature. After the appearance of either perithecia or conidiophores, spore masses were transferred from these structures to 2% Biolab malt-extract agar (MEA), and the cultures were purified.

For isolations from soil, 1 g of each sample was diluted in 100 mL sterile water. A dilution series with five dilutions was made. Of each of the dilutions, 1 mL was plated onto 2% malt- and 0.2% yeast-extract agar (MYA). The plates were incubated at 20 C for 1–3 d. Colonies with a *Sporothrix*-like appearance were transferred to clean MEA plates and purified. Isolates from bark beetles and humans were obtained following the methods described by Hsiau and Harrington (1997) and Vismer and Hull (1997), respectively.

O. ulmi (Buisman) Nannf. and *O. ips* (Rumbold) Nannf. isolates included as outgroups in the phylogenetic analysis have been sequenced as part of an earlier study (Harrington et al 2001).

All isolates in this study are maintained on MEA slants at 4 C in either the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa, or in the culture collection (C) of T. C. Harrington, Department of Plant Pathology, Iowa State University, U.S.A. (TABLE I).

DNA sequencing and sequence analysis.—To conduct phylogenetic analyses, isolates were grown 10 d in a liquid medium containing 2% malt extract. DNA was extracted using the method of DeScenzo and Harrington (1994). A part of the ribosomal DNA operon, including the 3′ end of the small subunit (SSU) rDNA gene, internal transcribed spacer (ITS) region 1, the 5.8S rRNA gene, ITS region 2 and the 5′ end of the 26S large subunit rDNA gene (LSU), was amplified using PCR with the primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al 1990). The reaction mixture (50 μL final volume) contained 2.6 U Expand™ High Fidelity *Taq* Polymerase mixture (Boehringer Mannheim, South Africa), 5 μL PCR reaction buffer, 3 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.2 mM of each primer. PCR reactions were performed in a Hybaid Touchdown PCR machine (Hybaid, Middlesex, UK). PCR conditions were: one cycle of 2 min at 95 C, followed by 40 cycles of 30 s at 95 C, 30 s at 55 C and 1 min at 72 C, followed by one cycle of 8 min at 72 C. PCR products were visualized by electrophoresis on a 1% (w/v) agarose gel, stained with ethidium bromide.

PCR fragments were purified using the QIAquick PCR purification kit. Both strands of the PCR fragments were sequenced using the primers ITS1-F, ITS4, CS2 and CS3 (Wingfield et al 1996) and the Thermo Sequenase Dye Terminator Cycle Sequencing Premix Kit (Amersham Life Science). Sequences were determined with an ABI Prism 377 Automatic DNA sequencer (Perkin Elmer).

The nucleotide sequences were aligned manually and the phylogenetic analyses performed using PAUP (phylogenetic analysis using parsimony) 4.0b2a (Swofford 1998). Uninformative characters were excluded and a heuristic search, us-

TABLE I. Isolates used in this study

Species	Isolate[a]	Other numbers	GenBank	Collector or supplier	Substrate	Origin
Ophiostoma abietinum	C696[b]	CBS125.89	AF484453	JG Marmolejo	*Abies vejari*	Mexico
O. albidum	C1190	CBS798.73	AF484475	A Mathiesen-Kåårik	wood	Sweden
O. ips	C327	—	AF198244	TC Harrington	—	USA, New York
O. narcissi	C1648	IMI349579	AF484451	—	*Narcissus* sp.	UK
O. nigrocarpum-like	CMW7619	C140	—	D Owen	*Pinus ponderosa*	USA, California
	CMW7620	C190	AF484473	D Owen	*Pinus ponderosa*	USA, California
	CMW7621	C201; ATCC22391; RWD237	AF484474	RW Davidson	*Dendroctonus* sp.	USA, California
	C314	CBS408.77; RWD873	AF484452	HS Whitney	*Pinus ponderosa*	USA, California
	C349	—	—	TC Harrington	*Pinus taeda*	USA, Louisiana
	C558	—	—	TC Harrington	*Dendroctonus frontalis*	USA, Mississippi
	C818	—	—	Y Masuya	*Quercus serrata*	Japan
	C946	—	—	R Blanchette	—	New Zealand
	C1142	13NZ-493	—	R Farrell	—	New Zealand
	C1302	—	—	D McNew	*Pinus radiata*	USA, California
	CMW1468	C1211	AF484457	Y Hiratsuka, Y Yamaoka	*Dendroctonus ponderosae*	Canada, British Columbia
	CMW2543	—	—	PW Crous	*Eucalyptus* leaves	South Africa, Western Cape
	CMW7131	HA206	—	E Halmschlager, T Kirisits	*Quercus petraea*	Austria
O. ponderosae	C87[b]	ATCC26665; RWD900	AF484476	TE Hinds	*Pinus ponderosae*	USA, Arizona
O. stenoceras	C962	UFV177	AF484454	A Alfenas	*Eucalyptus globulus*	Uruguay
	C965	3NZ-35	—	R Farrell	sapwood	New Zealand
	C966	3NZ-38b	AF484455	R Farrell	wood	New Zealand
	C982	—	—	R Farrell	leaves of conifer	New Zealand
	C1189	CBS360.71; UAHM5131	AF484447	F Mariat	skin of human head	France
	C1191	CBS208.75	AF484448	D Herderschee	skin of human	Netherlands
	C1192	CBS103.78	AF484449	RW Davidson	human	USA, Chicago
	C1193	CBS470.92	AF484450	F Marziano	soil	Italy
	CMW129	C80; RWD905; CO459	AF484456	RW Davidson	—	USA
	CMW2344	—	—	GHJ Kemp	*Eucalyptus smithii*	South Africa, Kwazulu-Natal
	CMW2347	—	—	GHJ Kemp	*Eucalyptus fastigata*	South Africa, Mpumalanga
	CMW2348	C703	—	GHJ Kemp	*Eucalyptus smithii*	South Africa, Kwazulu-Natal
	CMW2349	—	AF484458	GHJ Kemp	*Eucalyptus grandis*	South Africa, Mpumalanga

TABLE I. Continued

Species	Isolate[a]	Other numbers	GenBank	Collector or supplier	Substrate	Origin
Ophiostoma stenoceras	CMW2524	—	AF484459	ZW de Beer	*Acacia mearnsii*	South Africa, Kwazulu-Natal
	CMW2530	—	AF484460	ZW de Beer	*Eucalyptus grandis*	Colombia
	CMW2533	—	—	ZW de Beer	*Eucalyptus grandis*	Colombia
	CMW2625	C447; UCB57.013	AF484461	ML Berbec, JW Taylor	—	USA
	CMW3202[b]	C1188; CBS237.32	AF484462	H Robak	pine pulp	Norway
	CMW3998	—	AF484463	VN Thanh	soil ex. *Euc.* plantation	Kenya
	CMW4003	—	—	VN Thanh	soil ex. *Euc.* plantation	Kenya
	CMW4007	—	AF484464	VN Thanh	soil ex. *Euc.* plantation	Colombia
	CMW4020	—	—	VN Thanh	soil ex. *Euc.* plantation	Colombia
	CMW4031	—	AF484465	ZW de Beer	indigenous hardwood	Indonesia
	CMW5346	—	AF484466	A Smit	canker on apple tree	South Africa, Western Cape
	CMW5347	—	—	A Smit	canker on apple tree	South Africa, Western Cape
Ophiostoma ulmi	C1182	CBS102.63	AF198232	FW Holmes, HM Heybroek	*Ulmus hollandica*	Netherlands
Sporothrix schenckii	CMW7132	—	AF484467	JJ van der Merwe	human	South Africa
	CMW7133	—	AF484468	ZW de Beer	*Rosa* sp.	South Africa
	CMW7611	MRC6856	AF484469	HF Vismer	human sporotrichosis	South Africa
	CMW7612	MRC6862	—	HF Vismer	human sporotrichosis	South Africa
	CMW7613	MRC6864	AF484470	HF Vismer	human sporotrichosis	South Africa
	CMW7614	MRC6867	—	HF Vismer	human sporotrichosis	South Africa
	CMW7615	MRC6956	—	HF Vismer	human sporotrichosis	South Africa
	CMW7616	MRC6957	—	HF Vismer	human sporotrichosis	South Africa
	CMW7617	MRC6963	AF484471	HF Vismer	soil	South Africa
	CMW7618	MRC6965	AF484472	HF Vismer	soil	South Africa
	—	ATCC14284	AF117945	CW Emmons	human	USA, Maryland

[a] C = Culture Collection of T.C. Harrington, Department of Plant Pathology, Iowa State University, Iowa, USA. CMW = Culture Collection Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[b] Isolates associated with the holotype.

ing TBR (Tree Bisection and Reconstruction) branch swapping (MULPAR on), was conducted to determine the most-parsimonious trees. Trees were rooted with sequences of *O. ulmi* and *O. ips*. One thousand bootstrap analyses were run to determine confidence levels at the branching points. Aligned data and tree were deposited at TreeBASE (Study accession number = S788; Matrix accession number = M1248).

RESULTS

Sequence analysis.—PCR products of the isolates of ingroup species were approximately 530 bp in size. Within the ITS 1 region of all isolates, there was a GC rich area of approximately 70 bp. This area proved difficult to sequence, probably due to GC folding, resulting in secondary structures that would be difficult for the polymerase to read through. *S. schenckii* isolates proved to be the most difficult to sequence. Although it was possible to get the full sequence for most of the isolates, there were five isolates for which approximately 25 bases could not be determined. Manual alignment of the dataset resulted in a total of 617 characters, including gaps. From the GC rich area in ITS 1, 61 characters were excluded from the analyses. Of the remaining 556 unordered characters, 353 were constant, 89 variable characters were parsimony uninformative, leaving 114 informative characters in the analyses. Most of the variation in the sequence data was found within the ITS 1 region.

Using *O. ulmi* and *O. ips* as outgroup taxa, 80 most-parsimonious trees (CI = 0.828, HI = 0.172, RI = 0.937) of 308 steps were produced. Four main clades were resolved in all trees. Variation among the trees resulted from minor branch alternatives within the main clades. A single tree was chosen for presentation (FIG. 1). Bootstrap values supporting the branches of groups *O. stenoceras* and *O. nigrocarpum* were 100% and 90% respectively. The two clades of the *S. schenckii* complex each were supported with 100% confidence.

DISCUSSION

In this study, we could show that *O. stenoceras*, *O. nigrocarpum* and *S. schenckii* are closely related phylogenetically. The rDNA sequence data, however, clearly separated these three species. Our results support earlier suggestions (Berbee and Taylor 1992) that *S. schenckii* could be classified within the teleomorph genus *Ophiostoma*. They also support previous morphological, biochemical and molecular studies that have separated *O. stenoceras* and *S. schenckii* (Travassos and Lloyd 1980, Summerbell et al 1993). Another interesting outcome of this study is that, although distinct, *O. nigrocarpum* and *O. stenoceras* appear to be more closely related to each other than to *S. schenckii*. Furthermore, it appears that *S. schenckii* represents more than one species. The four main clades in the phylogenetic tree (FIG. 1) represent *O. stenoceras*, *O. nigrocarpum*, and two separate groups containing isolates of *S. schenckii*.

Ophiostoma stenoceras *clade.*—The *O. stenoceras* clade includes 29 isolates, including the strain representing the type of the species (CMW3202) from Norway. Other *O. stenoceras* isolates from Europe (Italy, Netherlands and France), U.S.A. and New Zealand, as well as isolates resembling *O. stenoceras* from Africa, South America and Indonesia, grouped together in this clade. This study thus represents the first report of *O. stenoceras* from Colombia, Kenya, Uruguay, Indonesia and South Africa. In South Africa the fungus is distributed widely on a variety of hardwood hosts.

The fact that three of the *O. stenoceras* isolates in the study came from humans is of particular significance. The isolate of Mariat (C1189) from healthy human scalp, and which was suggested to represent the teleomorph of *S. schenckii* (Mariat 1971a), grouped clearly within the *O. stenoceras* clade. Our data, therefore, confirm previous studies showing that this isolate cannot be considered the teleomorph of *S. schenckii* (Mendonça-Hagler et al 1974, Suzuki et al 1988).

The *O. albidum* isolate (C1190) grouped within the *O. stenoceras* clade. This species originally was described from bark beetle galleries in Sweden and was distinguished from *O. stenoceras* by its smaller perithecia (Mathiesen-Kåårik 1953). Although other slight morphological differences between the two species have been reported (Kåårik 1960, Mathiesen-Kåårik 1960, Aoshima 1965, Griffin 1968), De Hoog (1974) and Upadhyay (1981) treated *O. albidum* as a synonym of *O. stenoceras*. Our results support this synonymy.

The isolate of *O. ponderosae* (C87) also grouped in the *O. stenoceras*-clade, and the sequence was identical to that of the strain representing the type of *O. stenoceras*. In the original description of *O. ponderosae*, Hinds and Davidson (1975) reported ascospores of 4.5 to 5.5 μm long, while Robak (1932) reported ascospore lengths of 2.0–2.9 μm in the original description of *O. stenoceras*. In subsequent descriptions of *O. stenoceras*, however, the range of ascospore lengths was expanded to include lengths of up to 5.5 μm (Davidson 1942, Aoshima 1965, Upadhyay 1981). *Ceratocystis ponderosae* (= *O. ponderosae*) was treated by Upadhyay (1981) as a synonym of *O. populinum*

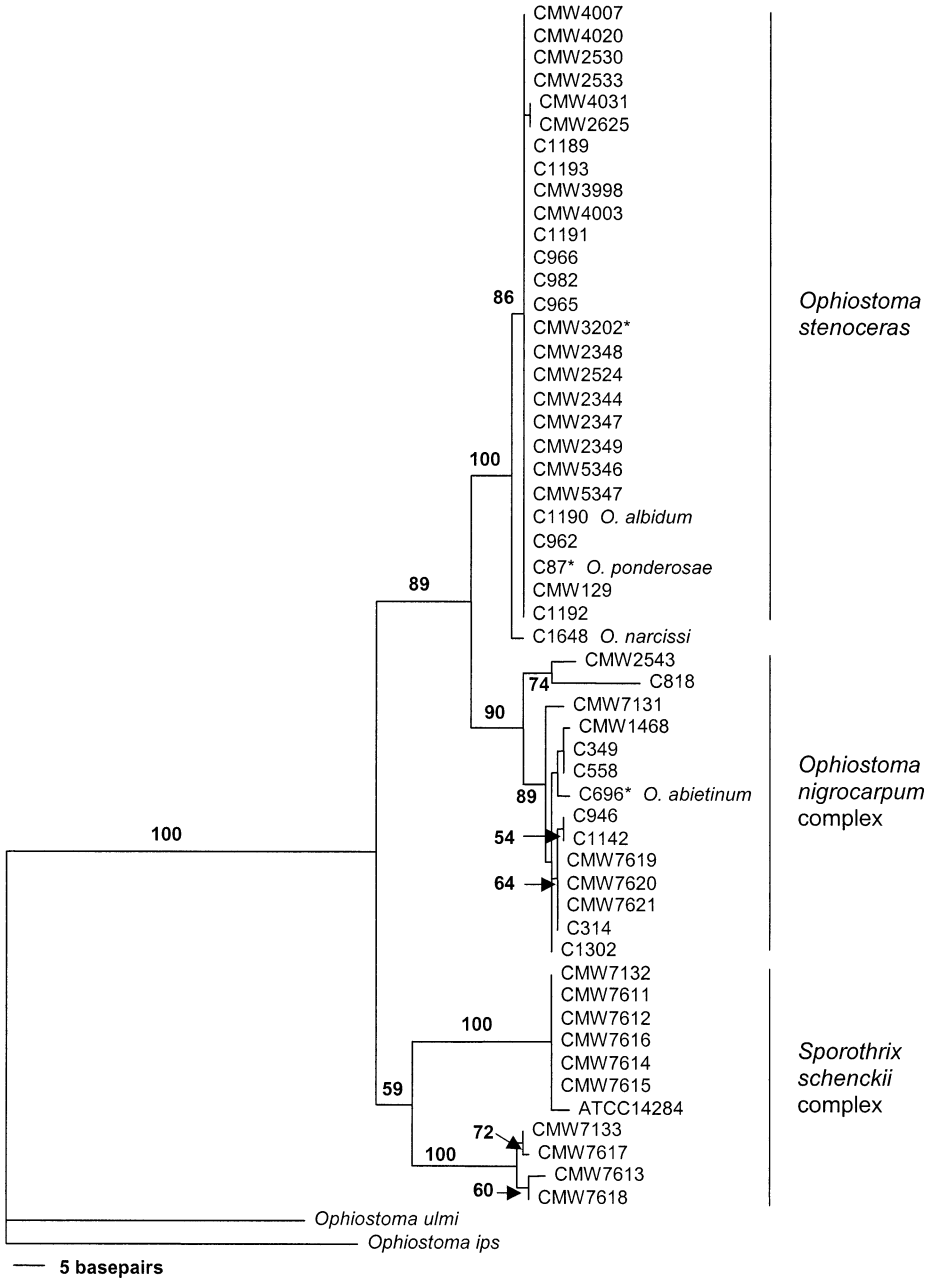

FIG. 1. One of the most-parsimonious trees obtained by heuristic searches of the partial ribosomal RNA operon (including partial small subunit, internal transcribed spacer (ITS1) region, 5.8S gene, ITS2, and partial large subunit). Bootstrap values are above the lines at branching points. Asterisks (*) indicate isolates associated with the type material.

(Hinds & Davidson) de Hoog & Scheffer. Hausner et al (1993), however, suggested that this synonymy might not be valid, based on partial LSU rDNA sequence data. They reinstated the species and transferred it to the genus *Ophiostoma* (Hausner et al 1993). Our results suggest that *O. ponderosae* is a synonym of *O. stenoceras*.

The isolate of *O. narcissi* (C1468) also grouped in the *O. stenoceras* clade but differed by 4 bp from other isolates in the group. Limber (1950) mentioned differences in perithecial size and ascospore shape between the two species, and Hunt (1956), De Hoog (1974), Olchowecki and Reid (1973) and Upadhyay (1981) also treated them as separate species. *Ophiostoma narcissi* originally was isolated from *Narcissus* bulbs in the Netherlands and has been found on *Narcissus* bulbs in the United Kingdom (isolate used in this study), New Zealand (Laundon 1973), Canada (Olchowecki and Reid 1973) and the U.S.A. (Upadhyay 1981). *Ophiostoma stenoceras* typically is isolated

from woody substrates and, as was shown in this study, from soil. Although the base-pair differences between *O. narcissi* and the *O. stenoceras* isolates might not seem sufficient to distinguish between the species phylogenetically, we believe that the morphological and ecological differences indicate that the two species are distinct. We, therefore, suggest that *O. narcissi* should be considered distinct from *O. stenoceras*, until further molecular data of more isolates become available.

The Ophiostoma nigrocarpum *clade.*—This clade contained 14 isolates, which were divided into two smaller clades, each with significant bootstrap support. The larger of the two clades included eight authenticated isolates of *O. nigrocarpum* from pines and bark beetles in the U.S.A. and Canada. Two *Sporothrix* isolates from New Zealand (C946 and C1142) also grouped within the main *O. nigrocarpum* clade, as did the strain associated with the type of *O. abietinum* (C696). *Ophiostoma abietinum* originally was described from *Abies* in Mexico (Marmolejo and Butin 1990) and can be considered an intermediate between *O. stenoceras* and *O. nigrocarpum*, based on perithecium morphology (Robak 1932, Davidson 1966, Marmolejo and Butin 1990). The remaining isolate (CMW7131) in the larger *O. nigrocarpum* clade originated from *Quercus* in Austria.

The second, smaller clade within the *O. nigrocarpum* group consists of a South African isolate from *Eucalyptus* (CMW2543) and a Japanese isolate from *Quercus* (C818). Since both clades in the *O. nigrocarpum* group exhibit some variability, we consider this group a poorly understood species complex. *Ophiostoma abietinum*, therefore, still should be treated as a distinct species until further studies have been conducted.

Sporothrix schenckii *clades.*—Within the larger *S. schenckii* clade, there are two strongly supported groups of isolates. All isolates from the first group originated from diseased human tissue. This includes the American isolate from a human (ATCC14284), which groups with the South African isolates from humans. With one exception (CMW7613), all the isolates from the second group originated either from soil or plant material. This confirms previous observations in which morphological and physiological differences were observed among isolates of *S. schenckii* from human tissue and those from other sources (Travassos and Lloyd 1980). Isolates from wood and soil also tend to be less pathogenic to mice (Travassos and Lloyd 1980), suggesting some differences between them. Whether these two groups of isolates represent distinct species needs further evaluation, as does the origin of the human pathogen.

In this study the phylogenetic relationships between *O. stenoceras* and *S. schenckii* were resolved. The study also highlighted the need for additional investigations of the *O. nigrocarpum* complex. Additional isolates and other regions of the genome should be included in such studies. The two clades that are resolved in the *S. schenckii* group are intriguing and need further investigation, both with more sequences and clinical trials.

ACKNOWLEDGMENTS

We thank the Foundation of Research Development (FRD) and the members of the Tree Pathology Programme (TPCP), South Africa, for financial support. We are grateful to various colleagues listed in TABLE I for supplying cultures. Doug McNew and Joe Steimel of Iowa State University also made valuable contributions to this study in the identification of cultures and in DNA sequencing.

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