Mycologia, 96(4), 2004, pp. 866–878.
© 2004 by The Mycological Society of America, Lawrence, KS 66044-8897

Two new *Ophiostoma* species with *Sporothrix* anamorphs from Austria and Azerbaijan

Dilzara N. Aghayeva[1,2]
Michael J. Wingfield

Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002 South Africa

Z. Wilhelm de Beer

Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002 South Africa

Thomas Kirisits

Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, BOKU-University of Natural Resources and Applied Life Sciences, Vienna, Hasenauerstrasse 38, A-1190 Vienna, Austria

Abstract: The genus *Ophiostoma* includes numerous species of primarily insect-vectored, wood-staining fungi. Several anamorph genera that differ in their micronematous or macronematous conidiogenous cells have been associated with *Ophiostoma* species. Among the former group, *Sporothrix* is associated with many species and is characterized by conidiogenous cells that arise laterally or terminally from any place on the hyphae and produce nonseptate conidia on sympodially developing denticles. The purpose of this study was to characterize ophiostomatoid isolates with *Sporothrix* anamorphs recently collected in Austria and Azerbaijan. The isolates were characterized based on comparisons of rDNA and β-tubulin sequence data. Morphology, growth in culture, and sexual reproductive mode were also considered. Phylogenetic analyses of the combined sequence data showed that the isolates formed two distinct groups, one including isolates from Austria and the other isolates from Austria and Azerbaijan. Growth at 25 C and morphology revealed some differences between the two groups, and supported the view that they represent two new species, which we describe here as *Ophiostoma fusiforme* sp. nov. and *Ophiostoma lunatum* sp. nov. Both these groups phylogenetically were related to, but distinct from, *Ophiostoma stenoceras.*

Accepted for publication November 17, 2003.

[1] Corresponding author. E-mail: adilzara@hotmail.com

[2] Permanent address: Institute of Botany, ANAS, Patamdar sh. 40, Baku 370073, Republic of Azerbaijan.

Key words: DNA sequences, *fusiforme*, *lunatum*, *nigrocarpum*, phylogeny, sap staining, *stenoceras.*

INTRODUCTION

The ophiostomatoid fungi are a group of wood-inhabiting and insect-associated ascomycetes with a worldwide distribution. These fungi are characterized by ascomata with spherical bases and long necks that give rise to gloeoid masses of ascospores (Hunt 1956, Upadhyay 1993, Wingfield et al 1993). The genus *Ophiostoma* H. & P. Sydow includes numerous species that are associated with bark beetles, and some of these are forest pathogens. Others cause gray, black or brown discoloration of wood, which can lead to substantial losses in lumber value (Seifert 1993). Of the plant pathogenic species of *Ophiostoma,* the Dutch elm disease fungi are the most important. *Ophiostoma ulmi* and *O. novo-ulmi* have been responsible for two pandemics of Dutch elm disease and *O. ulmi* later was replaced by *O. novo-ulmi,* a more aggressive pathogen (Brasier 2000, Pipe et al 2000).

Ophiostoma has many anamorphs that have been classified in at least four genera: *Pesotum* Crane & Schoknecht, *Leptographium* Lag. & Melin, *Hyalorhinocladiella* Upadh. & Kendr. and *Sporothrix* Hekt. & Perk. These genera are distinguished based on differences in conidiophore structure and in patterns of conidial development (Seifert and Okada 1993, Benade et al 1995, 1997, Okada et al 1998, Jacobs and Wingfield 2001). *Pesotum* accommodates anamorphs producing synnemata (Okada et al 1998), while *Leptographium* is characterized by dark mononematous conidiophores that give rise to a series of branches, terminating in conidiogenous cells (Jacobs and Wingfield 2001). *Hyalorhinocladiella* species lack the distinct denticles on conidiogenous cells that are typical for *Sporothrix* species (Benade et al 1996), however, the distinction between *Hyalorhinocladiella* and *Sporothrix* based on morphology is not clear because intermediate forms between the two genera exist (Mouton et al 1992, Benade et al 1996, 1997).

De Hoog (1974) described *Sporothrix* as an artificial genus for species morphologically similar to the type species, *S. schenckii* Hekt. & Perk. Based on his description, conidiogenous cells that arise laterally or terminally from any place on the hypha, bearing one-

celled conidia on sympodially developing denticles, characterize the genus. However, it is generally accepted that the genus is paraphyletic and includes species of both ascomycetes and basidiomycetes (de Hoog 1993). Thus, *Sporothrix* species associated with species of *Ophiostoma* represent only one of a number of morphologically similar but phylogenetically different lineages (Middelhoven et al 2000).

Recent collections of ophiostomatoid fungi from Azerbaijan and Austria include a number of *Ophiostoma* isolates with *Sporothrix* anamorphs. Preliminary morphological observations of these fungi have shown that they are broadly similar to *O. stenoceras* (Robak) Melin & Nannf. and *O. nigrocarpum* (Davidson) de Hoog. The aim of this study was to characterize these isolates based on morphology and DNA sequence comparisons and thereby determine their phylogenetic relationships.

MATERIALS AND METHODS

Isolates.—Seven isolates from Azerbaijan and Austria were compared with isolates of *O. stenoceras* and *O. nigrocarpum* used in previous studies (Davidson 1966, de Beer et al 2003) (TABLE I). The ex-type cultures of both species were included (TABLE I). Isolates (CMW7612, CMW7614, CMW7615) of *S. schenckii,* one isolate (CMW1468) previously described as belonging to the *O. nigrocarpum*-complex (de Beer et al 2003) and two isolates (CMW109, CMW110) morphologically similar to the isolate CMW1468, were included. In total, 20 isolates from both hardwoods and conifers from different geographic locations were included in this study. These isolates are all maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Representative isolates also have been deposited in the collection of the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Herbarium specimens linked to key cultures have been deposited in the herbarium of the National Collection of Fungi, Pretoria, South Africa (PREM).

DNA sequencing and sequence analysis.—Isolates were grown 10 d on 2% malt-extract agar (MEA: 20 g malt extract [Biolab, Merck], 20 g agar [Biolab, Merck], 1L dH_2O). Approximately 10 mg of mycelium was scraped from the surface of actively growing cultures using a sterile scalpel. The mycelium was transferred to Eppendorf tubes, suspended in 200 μL PrepMan Ultra Sample Preparation reagent (Applied Biosystems), ground gently with a glass rod, incubated 10 min at 95 C and centrifuged at 13 000 rpm for 5 min. One hundred μL supernatant of the aqueous phase was used directly for PCR. The internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal DNA operon, including the 5.8S gene, were amplified using primers ITS1-F and ITS4 (White et al 1990). Part of the β-tubulin gene was amplified using primers Bt2a and Bt2b (Glass and Donaldson 1995). The reaction mixture (50 μL final volume) contained 2.5 U Super-Term DNA Polymerase mixture (Hoffmann-La Roche, U.S.A.), 1× PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTPs and 0.2 mM of each primer. PCR reactions were performed in a thermal cycler (Hoffmann-La Roche). PCR conditions were: one cycle of 2.5 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, and a final cycle of 8 min at 72 C. PCR products were electrophoresed on a 1% (w/v) agarose gel stained with ethidium bromide and the amplicons visualized under UV light. PCR fragments were purified using the High Pure PCR Product Purification Kit (Roche). Both strands of the PCR products were sequenced using the above-mentioned primers and the ABI Prism® BigDye™ Terminator version 3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems). DNA sequencing reactions were analyzed on the ABI PRISM®3100 Genetic Analyzer or the ABI PRISM™ 377 DNA sequencer.

The sequences were aligned manually using Sequence Navigator and analyzed with Phylogenetic Analysis Using Parsimony (PAUP) 4.0b (Swofford 1998). Phylogenetic analysis was done first for each gene region separately, followed by an analysis of the combined data set of the ITS and β-tubulin sequences. A partition homogeneity test in PAUP was performed to determine the congruence and combinability of the two sequence data sets. Uninformative characters were excluded, and a heuristic search, using TBR branch swapping (MULPAR on), was used to determine the most parsimonious trees. Trees were rooted using sequences of *S. schenckii* (de Beer et al 2003). Confidence intervals of the branch points were determined by 1000 bootstrap analyses.

Morphological studies.—Isolates were grown 10 d on 2% MEA. To stimulate sexual sporulation, 2% water agar (WA: 20 g agar, 1L dH_2O) amended with debarked oak and pine twigs (40 mm long × 5 mm wide) or with milled elm and spruce sapwood (25 g milled sapwood, 7.5 g agar, 250 mL water), as described by Brasier (1981), was used. Cultures were incubated at room temperature for 10 d. Colony colors were determined using the color charts of Rayner (1970).

Fifty measurements were made of each taxonomically informative structure in isolates that produced perithecia. Three-day-old slide cultures, mounted in lactophenol, were prepared to study the anamorph structures (Riddell 1950).

Growth in culture.—Agar disks (5 mm diam) bearing mycelium of selected isolates (TABLE I) were transferred from the actively growing margins of 1 wk old cultures and placed at the center of Petri dishes containing 20 mL 2% MEA. Isolates were grown at temperatures of 5–35 C at 5 C intervals for 10 d in the dark. Six measurements of colony diameter were made for each isolate, by taking two measurements from each of three replicate cultures. Results were computed as an average of these six measurements. Isolates that did not grow at high temperatures were subsequently maintained at room temperature to test their viability.

Mode of sexual reproduction.—Ten single ascospore and 10 single conidial cultures were prepared for each isolate (CMW9968, CMW8281, CMW8285, CMW7131, CMW10565,

TABLE I. Isolates used in this study

Species	Isolates[a]	Other numbers[b]	Substrate	Origin	Collector or suppler	Mode of sexual repro-duc-tion[f]	Growth[g]	GenBank numbers	
								ITS	β-tubulin
O. fusiforme	CMW9968	CBS112912	*Populus nigra*	Azerbaijan	DN Aghayeva	SF	T	AY280481	AY280461
	CMW8281	CBS112909	*Castanea sativa*	Azerbaijan	DN Aghayeva	SF	NT	AY280482	AY280462
	CMW8285	CBS112910	*Castanea sativa*	Azerbaijan	DN Aghayeva	SF	NT	AY280483	AY280463
	CMW7131	CBS112925, HA206	*Quercus petraea*	Austria	E Halmschlager	SF	T	AY280497	AY280464
	CMW10565	CBS112926, CTK102	*Larix decidua*	Austria	T Kirisits	SF	T	AY280484	AY280465
O. lunatum	CMW10563	CBS112927, CTK101	*Carpinus betulus*	Austria	T Kirisits	SF	T	AY280485	AY280466
	CMW10564	CBS112928, IC/P/CH/1	*Larix decidua*	Austria	T Kirisits	SF	T	AY280486	AY280467
Sporothrix sp.	CMW1468[e]	C1211	*Dendroctonus ponderosa*	Canada	Y Hiratsuka, Y Yamaoka	SS	T	AY484457	AY280468
	CMW109	C232	*Pinus echinata*	USA	F Hinds	SS	T	AY280487	AY280469
	CMW110	C235	*Pinus echinata*	USA	F Hinds	SS	T	AY280488	AY280470
O. nigrocarpum	CMW650[c,d]	CBS637.66	*Abies* sp.	USA	RW Davidson	SS	T	AY280489	AY280479
	CMW651[d]	CBS638.66	*Pseudotsuga menziesii*	USA	RW Davidson	SF	T	AY280490	AY280480
O. stenoceras	CMW3202[c,e]	C1188, CBS237.32	pine pulp	Norway	H Robak	SF	T	AY484462	AY280471
	CMW2344[e]	—	*Eucalyptus smithii*	South Africa	GH Kemp	SF	T	AY280491	AY280472
	CMW2524[e]	—	*Acacia mearnsii*	South Africa	ZW de Beer	SF	NT	AY484459	AY280473
	CMW11192[e]	C965	sap	New Zealand	R Farrell	NT	NT	AY280492	AY280474
	CMW11193[e]	C966	wood	New Zealand	R Farrell	NT	NT	AY280493	AY280475
S. schenckii	CMW7612[e]	MRC6862	Human sporothrichosis	South Africa	HF Vismer	SS	NT	AY280494	AY280476
	CMW7614[e]	MRC6867	Human sporothrichosis	South Africa	HF Vismer	SS	T	AY280495	AY280477
	CMW7615[e]	MRC6956	Human sporothrichosis	South Africa	HF Vismer	SS	T	AY280496	AY280478

[a] CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, SA.

[b] C = Culture collection of T.C. Harrington, Department of Plant Pathology, Iowa State University, USA; CBS = Culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CTK, HA, IC/P/CHl = Culture collection of the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, BOKU-University of Natural Resources and Applied Life Sciences, Vienna; MRC = Culture collection of PROMEC, Medical Research Council, Cape Town, SA.

[c] Ex-type cultures.

[d] Authentic cultures for *O. nigrocarpum* (Davidson 1966).

[e] Cultures used by De Beer et al (2003).

[f] Mode of sexual reproduction: SF—self fertile, SS—self sterile.

[g] Growth: T—tested, NT—not tested.

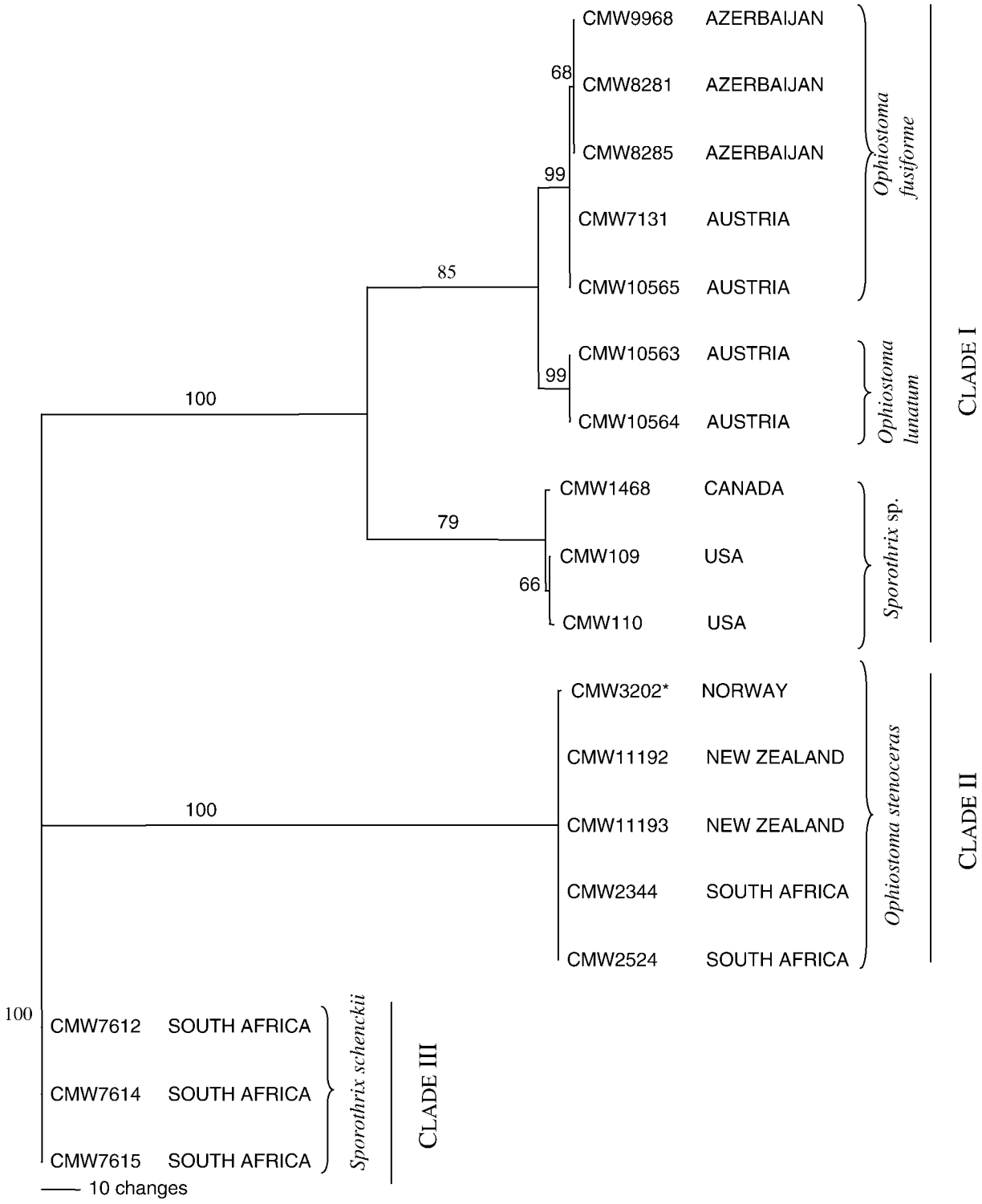

FIG. 1. One of the two most parsimonious trees obtained by a heuristic search of the combined data set of the 5.8S gene, including ITS1 and ITS2 rRNA operon regions, and the partial β-tubulin gene. Bootstrap values are indicated at the branching points. * Ex-type isolate.

TABLE II. Distinguishing characters for species of *Ophiostoma* and *Sporothrix* considered in this study

	Ophiostoma fusiforme	*Ophiostoma lunatum*	Robak (1932) *O. stenoceras*	Davidson (1966) *O. nigrocarpum*	De Hoog (1974) *Sporothrix schenckii*	*Sporothrix* sp.
Perithecia	starting to form in about 30 days, maturing quickly, sometimes with 2 necks	starting to form in about 40 days, maturing quickly, rarely with 2 necks	starting to form in about one week, maturing after week. Abnormal examples have up to five necks in agar	starting to form in about two weeks, maturing slowly	—	—
Base: diam.	121.5–273.8 μm	59.5–178.3 (–204.5) μm	130–250 μm	50–80 μm	—	—
Neck:						
length	301.8–985 (–1168) μm	162.4–554.2 (–700) μm	420–1500 μm	120–160 μm	—	—
width at base	21.8–33.7 (–44.9) μm	15.3–33.4 (–40.5) μm	20 μm	15–25 μm		
width at apex	9.1–13.5 (–18) μm	7.5–10.4 (–13.8) μm	8.0–12 μm	10–12 μm		
Ornamental hyphae:						
length	16.6–94.5 (–142.5) μm	11.9–106.8 μm	—	absent or very short, hyaline and curved outward	—	—
width	1.71–2.2 (–2.6) μm	0.69–2.6 (–3.58) μm				
Ostiolar hyphae:						
length	23.4–68.4 (–96.4) μm	13.6–56.9 (–61.7) μm	20–48 (–60) μm	—	—	—
width	1.2–2.5 (–3) μm	1.01–1.7 (–2.7) μm	2.6–2.7 μm			
Ascospores:						
length	3.4–4.3 (–5.4) μm	3.1–3.9 (–4.3) μm	2.0–2.9 μm	3.0–4.0 μm	—	—
width	0.8–1.3 (–1.6) μm	0.7–1.2 μm	1.3–1.4 μm	1–1.3 μm		
Color of culture	white becoming dull white or blackish after perithecia formation	white becoming dull white or blackish after perithecia formation	grayish or white, first slightly domed	light grey to dark grey as perithecia develop, no dark color in the mycelium or substrate	at first hyaline, later often becoming grayish or dull brown	white, later becoming creamy or slightly yellowish in the center
Diam. of colony in 10 d	33.5 mm*	31.5 mm*	29.9 mm*	30 mm; 59.3 mm*	6–22 mm 21 mm*	30.4 mm*
Texture of culture	smooth, finely floccose, aerial hyphae present	smooth, finely floccose, aerial hyphae present	quite flat, in which aerial hyphae rarely occur, surface covered by thick, porridge-like conidia	culture hyaline, surface often yeast-like	smooth, soon becoming finely floccose, velvety, lanose, or somewhat funiculose	smooth, later becoming floccose, in 30 d old cultures aerial hyphae with tufts in the centre

TABLE II. Continued

	Ophiostoma fusiforme	*Ophiostoma lunatum*	Robak (1932) *O. stenoceras*	Davidson (1966) *O. nigrocarpum*	De Hoog (1974) *Sporothrix schenckii*	*Sporothrix* sp.
Conidiogenous cells	14.3–53.9 (–72) × (0.9–) 1.2–1.8 (–2) μm	11.3–35.2 (–59.4) × 0.8–1.3 (–1.5) μm	—	—	10–40 × 0.7–1.5 (–2) μm	14.1–49.2 (–90.01) × 0.8–1.6 (–1.8) μm
Denticles	sharp, 0.3–1.2 μm	two types: inconspicuous 0.3–0.6 μm; cylindrical, up to 4.5 μm	—	—	0.5–1 μm	inconspicuous 0.2–0.6 μm
Conidia	guttuliform to fusiform 3.2–5.9 (–8.0) × 1.1–1.9 (–2.1) μm	curved, crescent shape 2.3–4.8 (–6.2) × 0.8–1.5 (–1.6) μm	broadly ellipsoidal, basally pointed 3.4–6.9 × 2.0–3.4 μm	broadly ellipsoidal, basally rounded 3.0–5.0 × 1.5–3.0 μm	broadly ellipsoidal, basally sharp pointed 2.5–5.5 (–8) × 1.5–2.5 (–3) μm	cylindrical, basally pointed 2.7–4.5 (–6.3) × 0.67–1.1 (–1.4) μm

* Our measurements of diameter of colony on MEA at 25 C.

CMW10563, CMW10564, CMW651, CMW3202, CMW2344, CMW2524) that produced perithecia. For isolates that did not produce perithecia, 10 single conidial cultures were prepared. Single-spore cultures were incubated at room temperature on MEA. Those producing perithecia were considered homothallic. The perithecia were observed using light microscopy to confirm the presence of typical ascospores. Isolates that did not produce perithecia were considered heterothallic. Crosses in all possible combinations were made between these cultures to confirm this observation. Each culture was crossed against itself as a control.

RESULTS

Sequence analysis.—Fragments of approximately 541 bp were obtained for 18 of the isolates. Two *O. nigrocarpum* isolates (CMW650 and CMW651) yielded fragments 572 bp in size. Manual alignment resulted in a data set of 551 characters for each of the 18 taxa included in the analysis.

PCR products obtained with primers Bt2a and Bt2b were approximately 286 bp in size for 15 of the isolates. The fragments obtained for the two *O. nigrocarpum* isolates were 517 bp long, and those for the three *S. schenckii* isolates were 434 bp long. Sequences of the 15 isolates, excluding *O. nigrocarpum* and *S. schenckii,* started with an incomplete 5′ end of an exon approximately 99 bp in length, followed by an intron with a variable number of base pairs: 84 in *O. nigrocarpum,* 61 in *O. stenoceras,* 79 in *S. schenckii* and 57 bp in the other 10 *Ophiostoma* isolates. This intron was followed by an incomplete 3′ end of an exon of 130 bp in most of the isolates.

Ophiostoma nigrocarpum and *S. schenckii* differed from the other isolates in that they contained additional introns of about 165 bp and 86 bp, respectively, within the first exon. Sequences for the *O. nigrocarpum* isolates, as well as the additional intron of the *S. schenckii* isolates, were excluded from the analysis because they could not be aligned unambiguously with those of the other isolates. Without the intron, the remaining sequences of the *S. schenckii* isolates could be aligned with the sequences of the other isolates in the study. Although sequences of the *O. nigrocarpum* isolates did not align well with other isolates, a BLAST search with the ITS sequences (data not shown) confirmed that these isolates belong to the genus *Ophiostoma.*

Separate analyses of the rDNA and β-tubulin gene regions resulted in trees with similar topologies. The partition homogeneity test for the combined data set confirmed a significant congruence between the data sets ($P = 1.0$). A phylogenetic analysis thus was performed on the combined ITS and β-tubulin data set containing 860 characters, of which 696 were constant, two parsimony uninformative and 162 parsi-

mony informative. *S. schenckii* sequences were used as outgroup, and a heuristic search resulted in 3568 rearrangements. Two most-parsimonious trees were retained, with a length of 196 steps (CI = 0.980, RI = 0.020, HI = 0.993). One of these trees is presented in FIG. 1.

Three main clades were evident in the tree (FIG. 1) based on the combined data. Two of the clades represented isolates of *O. stenoceras* (Clade II) and *S. schenckii* (Clade III) respectively. Clade I contained three subclades. The first subclade included *Ophiostoma* isolates from both Azerbaijan and Austria (CMW9968, CMW8281, CMW8285, CMW7131, CMW10565) and was supported by a bootstrap value of 99%. The second subclade contained two *Ophiostoma* isolates from Austria (CMW10563, CMW10564) and also had 99% bootstrap support. The third subclade represented the isolate from Canada previously assigned to the *O. nigrocarpum* complex (de Beer et al 2003) and two other isolates from the United States. These data suggest that there are three distinct taxa in Clade I.

Morphological studies.—Morphologically similar perithecia were produced by isolates from Austria and Azerbaijan in Clade I. However, those in the first subclade had longer necks and wider base diameters than those in the second subclade (TABLE II). The isolates in the third subclade (CMW1468, CMW109, CMW110) did not produce perithecia. Only three *O. stenoceras* (CMW3202, CMW2344, CMW2524) and one *O. nigrocarpum* isolate (CMW651) formed perithecia. In the *O. stenoceras* isolates, protoperithecia were observed after 2 wk but these matured very slowly. Perithecial necks of the *O. stenoceras* isolates were longer and narrower than those in the other groups of isolates. Perithecia of *O. nigrocarpum* isolates were considerably smaller than those in the other species.

Anamorph morphology, especially the shape and the size of conidia, differed between isolates grouping in the three subclades of Clade I (TABLE II). In isolates in the first subclade, the heads of the conidiogenous cells were generally swollen with distinct denticles and conidia were guttuliform to fusiform (FIG. 2D, E). The heads of conidiogenous cells of isolates in the second subclade were slightly swollen and denticles were small, inconspicuous and rarely long or cylindrical (FIG. 3D, E, G). Conidia were clavate or crescent shaped and thus very different to those of isolates in the first subclade. In isolates residing in the third subclade of Clade I, conidiogenous cells were similar to those of *O. stenoceras* (Clade II) and *S. schenckii* (Clade III) but conidia were cylindrical with pointed bases and narrower than those of the latter species. In *O. stenoceras* the conidia were broadly ellipsoidal with pointed bases. Conidiogenous cells of *O. nigrocarpum* usually are shorter and slightly rough. Conidia of the *O. nigrocarpum* isolate (CMW650) were broadly ellipsoidal but basally rounded and smaller than those of the isolates in the third subclade of Clade I and of the conidia of *O. stenoceras*.

Growth in culture.—All isolates, except the two of *O. nigrocarpum* (CMW650 and CMW651), grew at temperatures of 10–30 C. The *O. nigrocarpum* isolates commenced growth at 15 C on Day 4, and at 25 C these isolates grew twice as fast as the others, covering the Petri dishes after 8 d. *Ophiostoma nigrocarpum* isolates grew rapidly at 35 C, while none of the other isolates grew at that temperature. Growth rate of isolates of *O. stenoceras* and the isolates in Clade I did not differ much (TABLE II).

Isolates from Austria and Azerbaijan (first and second subclades of Clade I) differed in culture morphology from isolates in clades II and III but were indistinguishable from each other and those from Canada and the United States (third subclade of Clade I). *O. stenoceras* isolates were white, later slightly yellowish (''buff '' 19d), flat with sparse aerial hyphae in comparison with the isolates residing in Clade I. The *O. nigrocarpum* isolates produced gray, brownish colonies (''honey'' 19''), with obvious aerial mycelium. The colonies of *S. schenckii* were ''smoke grey'' (21''''f) and 40 d old cultures showed ''Isabella color'' (19''i) toward the margins, first smooth, later undulating at the centers of 3 wk old cultures. Tufts of mycelium arising from different parts of the 40 d old cultures and exudates were common in these cultures (TABLE II).

Mode of sexual reproduction.—All single conidial and single ascospore cultures obtained from the seven isolates from Austria and Azerbaijan were self fertile, producing perithecia with viable ascospores. The three *O. stenoceras* isolates and one *O. nigrocarpum* isolate (CMW651) also were self fertile (TABLE I). Single conidial cultures obtained from the remaining isolates did not produce perithecia, even when crossed with other cultures. These included the extype *O. nigrocarpum* isolate (CMW650), the Canadian isolate (CMW1468) previously assigned to the *O. nigrocarpum*-complex by de Beer et al (2003), two North American isolates (CMW109, CMW110) and the *S. schenckii* isolates.

Taxonomy.—Morphological and molecular characteristics support the placement of the isolates from Azerbaijan and Austria into two phylogenetic groups which were different from the *Sporothrix* isolates

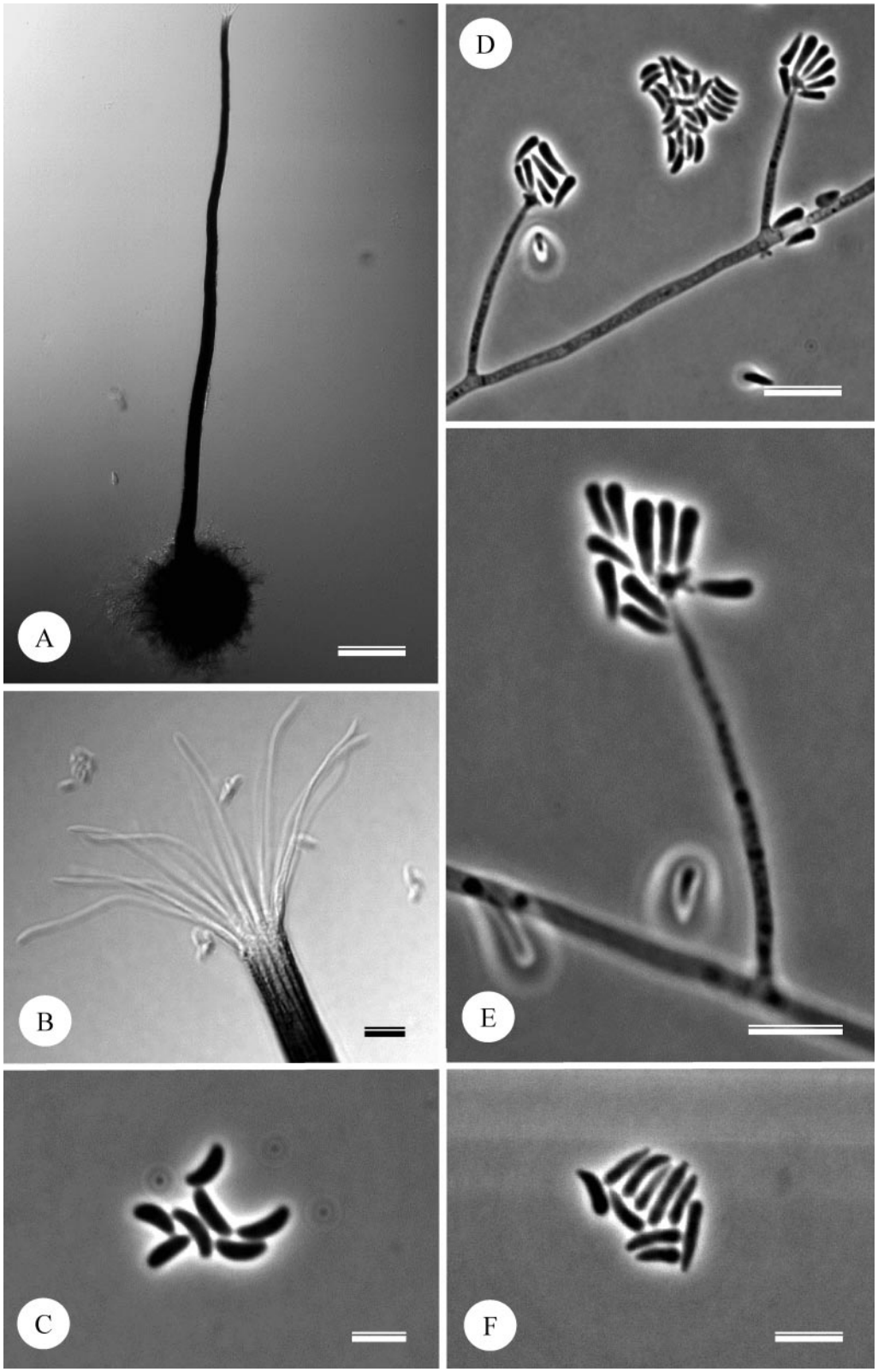

FIG. 2. Morphological characteristics of *Ophiostoma fusiforme*. A. perithecium with globose base and ornamental hyphae and long neck, B. ostiolar hyphae, C. allantoid ascospores in side view, D. conidiophores with plagiotropic branching, E. conidiogenous cell with conidia, F. fusiform conidia. Scale bars: A = 100 μm; B, D, E = 10 μm; C, F = 5 μm.

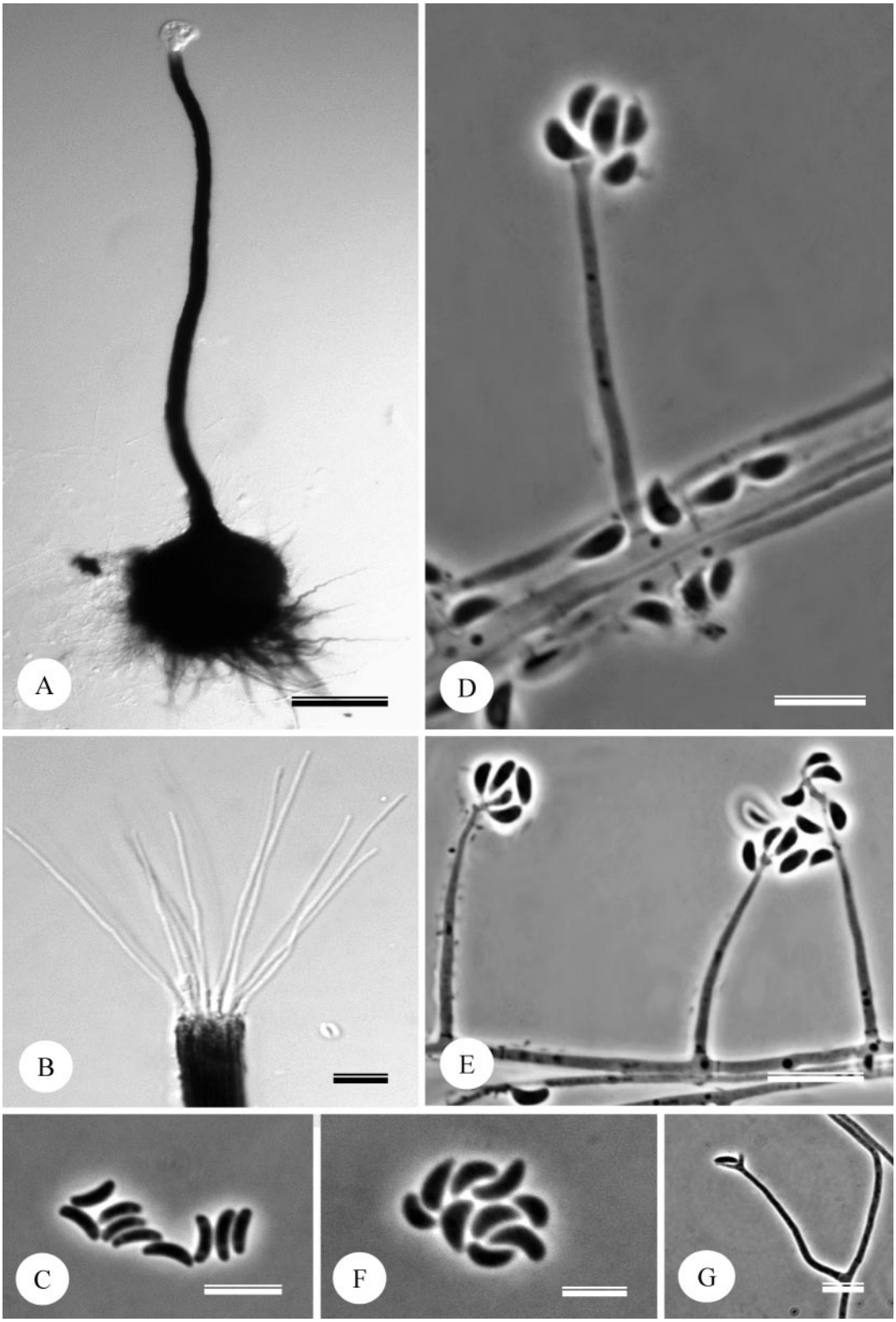

FIG. 3. Morphological characteristics of *Ophiostoma lunatum*. A. perithecium with globose base, ornamental hyphae and long neck, B. ostiolar hyphae, C. allantoid ascospores in side view, D. conidiogenous cell with denticles and conidia, E. conidiophores with orthotropic branching, F. crescent shape, curved conidia, G. conidiogenous cell with long denticles. Scale bars: A = 100 μm; B, D, E = 10 μm; C, F, G = 5 μm.

placed in the third subclade of Clade I. We consider subclades 1 and 2 to represent new taxa and herein described them as new species of *Ophiostoma*. Isolates in the third subclade did not produce perithecia in culture and, therefore, we have not provided a name for this fungus but refer to it as an undescribed species of *Sporothrix*.

Ophiostoma fusiforme D.N. Aghayeva & M. J. Wingfield sp. nov. FIG. 2

Anamorph. Sporothrix sp.

Coloniae in vitro in MEA post 10 dies in 25 C diametrum medium 33.5 mm attingentes; infra 10 C et supra 30 C non crescunt. Mycelium aereum primo laeve, mox floccosum cum orbibus concentricis incrementi. Incrementum in cultura parce rapidum, album, postea cinerascens vel post perithecia facta sunt nigrescens, superficie ima incolore vel sublutescente in coloniis veteris, aroma absente. Perithecia post 30 dies evoluta, superficialia vel in agaro semi-immersa. Bases globosae, nigrae, 121.5–273.8 μm diametro, trichomatibus hyphalibus laete brunneis vel brunneis, septatis, non ramosis, 16.6–94.5(–142.5) × 1.7–2.2(–2.6) μm, parietibus tenuibus vel crassis, ornatae. Colla interdum duo, ergo approximata, basin versus nigra, apicem versus laete brunnea, recta vel aliquando curvata, 301.8–985(–1168) μm longa, basi 21.8–33.7(44.9) μm, apice 9.1–13.5(–18) μm lata. Hyphae ostiolares multae, hyalinae, laeves, rectae vel subtortuosae, divergentes, 23.4–68.4(96.4) μm, basi 1.2–2.5(–3) μm latae. Ascosporae hyalinae, unicellulares, lateraliter visae allantoideae, 3.4–4.3(–5.4) × 0.8–1.3(–1.6) μm. Cellulae conidiogenae micronematosae, mononematosae, hyalinae, septatae, 14.3–53.9(–72) × (0.9–)1.2–1.8(–2) μm; parte apicale conidia formanti instar fasciculi globosi tumidi, cum denticulis acutis 0.3–1.2 μm longis. Conidia proxime in denticulis efferta, holoblastica, hyalina, unicellularia, clavata, plerumque fusiformia, interdum guttuliformia, basibus acutis, apicibus rotundatis, 3.2–5.9(–8.0) × 1.1–1.9 (–2.1) μm, singuli efferta, in massis mucosis aggregantes.

Colonies in vitro on MEA attaining an average diameter of 33.5 mm in 10 d at 25 C. No growth below 10 C or above 30 C. Aerial mycelium at first smooth, soon becoming floccose with concentric circles of growth. Growth in culture moderately rapid, white, later becoming dull white or blackish after perithecia form, reverse uncolored or slightly yellowish, (''Buff yellow— 19d'') in old cultures, aroma absent. Perithecia (FIG. 2A) developing after 30 d superficial or partly embedded in agar. Bases globose, black, 121.5–273.8 μm diam; ornamented with pale brown to brown, septate, thin- to thick-walled, unbranched hyphal hairs of variable length, 16.6–94.5(–142.5) × 1.7–2.2(–2.6) μm. Necks sometimes two, in this case close to each other, black at base, pale brown at apex, straight or occasionally curved, 301.8–985(–1168) μm long, 21.8–33.7(44.9) μm wide at base 9.1–13.5 (–18) μm at tip. Ostiolar hyphae (FIG. 2B) numerous, hyaline, smooth, straight or rather tortuous, divergent 23.4–68.4(96.4) μm, and 1.2–2.5(–3) μm wide at base. Ascospores hyaline (FIG. 2C), 1-celled, allantoid in side view, 3.4–4.3(–5.4) × 0.8–1.3(–1.6) μm. Conidiogenous cells (FIG. 2D, E) micronematous, mononematous, hyaline, septate, 14.3–53.9(–72) × (0.9–)1.2–1.8(–2) μm, apical part forming conidia consists of a globose, swollen cluster bearing sharp denticles 0.3–1.2 μm long. Conidia (FIG. 2F) produced directly on denticles, holoblastic, hyaline, 1-celled, clavate, guttuliform to fusiform (mostly fusiform, sometimes guttuliform) with pointed bases and rounded apices 3.2–5.9(–8.0) × 1.1–1.9(–2.1) μm, formed single, becoming aggregated in slimy masses.

Etymology. The Latin *fusiforme* refers to fusiform shaped conidia.

Specimens examined. AZERBAIJAN: Baku-Rostow highway. Wood of living *Populus nigra,* 03-VI-2000, *D.N. Aghayeva PREM57486 (culture CMW9968)* (HOLOTYPE). AUSTRIA. LOWER AUSTRIA: Hochleitenwald. Twig of living *Quercus petraea,* 07-IX-1992, *E. Halmschlager PREM57487 (culture CMW7131)*. AUSTRIA. STYRIA: Kindberg, Kindtalgraben near Troiseck. Galleries of the bark beetle *Ips cembrae* in the bark of *Larix decidua,* VII-1995, *T. Kirisits PREM 57488 (culture CMW10565)*. Herbarium specimens of holotype and paratypes have been deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

Ophiostoma lunatum D.N. Aghayeva & M. J. Wingfield sp. nov. FIG. 3

Anamorph. Sporothrix sp.

Coloniae in vitro in MEA post 10 dies in 25 C diametrum medium 31.5 mm attingentes; infra 10 C et supra 30 C non crescunt. Mycelium aereum primo laeve, mox floccosum cum orbibus concentricis incrementi, album, postea cinerascens vel post perithecia facta sunt subnigrescens, superficie ima incolore vel sublutescente, aroma absente. Perithecia post 40 dies evoluta, superficialia vel in agaro semi-immersa. Bases globosae, interdum pyriformae, nigrae, 59.5–178.3(–204.5) μm diametro, trichomatibus hyphalibus laete brunneis vel brunneis, septatis, non ramosis, longitudine variabili, 11.9–77.9(–106.8) × 0.69–2.6(–3.58) μm, parietibus tenuibus vel crassis, ornatae. Collum plerumque 1, interdum 2, basin versus nigrum, apicem versus laete brunneum, subundulatum vel curvatum, (162.4–)248.4 × 554.2(–700) μm longum, basi 15.3–33.4(– 40.5) μm, apice 7.5–10.4(–13.8) μm latum. Hyphae ostiolares multae, hyalinae, laeves, plerumque rectae, interdum curvatae, 13.6–56.9(–61.7) μm, basi 1.01–1.7(–2.7) μm latae. Ascosporae hyalinae, unicellulares, lateraliter visae allantoideae, 3.1–3.9 (–4.3) × 0.7–1.2 μm. Cellulae conidiogenae micronematosae, mononematosae, hyalinae, septatae, 11.3–35.2 (–59.4) × 0.8–1.3(–1.5) μm crassae, parte apicale cum denticulis, subincrassata. Denticula brevia, rotundata, interdum inconspicua, 0.3–0.6 μm longa, apicem versus cylindracea, usque ad 4.5 μm longa. Conidia proxime in denticulis efferta, et sympodialiter et lateraliter in hyphis unicis, holob-

lastica, hyalina, unicellularia, clavata, valde curvata, subfalcata, 2.3–4.8(–6.2) × 0.8–1.5(–1.6) μm, singuli efferta, in massis mucosis aggregantes. Blastoconidia lateralia copiosa.

Colonies in vitro on MEA attaining 31.5 mm diam in 10 d at 25 C. No growth below 10 C or above 30 C. At first smooth, soon becoming floccose with concentric circles. White, later becoming dull white or blackish after perithecial formation, reverse uncolored or slightly yellowish (19d and 19''b), aroma absent. Perithecia (FIG. 3A) developing after 40 d superficial or partly embedded in the agar. Bases globose, occasionally pyriform, black, 59.5–178.3 (–204.5) μm diam; ornamented with pale brown to brown, septate, thin- to thick-walled, unbranched hyphal hairs of variable length, 11.91–77.9(–106.84) × 0.69–2.6(–3.58) μm. Neck usually 1, rarely 2, black at base, pale brown at apex, slightly waved or curved, (162.4–)248.4 × 554.2(– 700) μm long, 15.3–33.4 (– 40.5) μm wide at base, 7.5–10.4(– 13.8) μm at tip. Ostiolar hyphae (FIG. 3B) numerous, hyaline, smooth, curved or rather straight, 13.6–56.9(–61.7) μm, and 1.01–1.7(–2.7) μm wide at the base. Ascospores (FIG. 3C) hyaline, 1-celled, allantoid in side view, 3.1–3.9(–4.3) × 0.7–1.2 μm. Conidiogenous cells (FIG. 3D, E, G) micronematous, mononematous, hyaline, septate, 11.3–35.2(–59.4) × 0.8–1.3(–1.5) μm thick, apical part bearing denticles, slightly swollen. Denticles short, rounded, sometimes inconspicuous 0.3–0.6 μm long, and cylindrical in apical region, up to 4.5 μm long. Conidia (FIG. 3F) produced directly on denticles, sympodially as well as laterally on single hyphae, holoblastic, hyaline, 1-celled, clavate, distinctly curved, somewhat crescent shaped 2.3–4.8(–6.2) × 0.8–1.5(–1.6) μm, formed singly, becoming aggregated in slimy masses. Lateral blastoconidia abundant.

Etymology. The Latin *lunatum,* refers to the lunate shaped conidia.

Specimens examined. AUSTRIA. VIENNA: Lainzer Tiergarten forest reservation Johannser Kogel. Inner bark of wind broken *Carpinus betulus,* 07-V-1995, *T. Kirisits PREM57489 (culture CMW10563)*(HOLOTYPE). AUSTRIA. STYRIA: Kindberg, Kindtalgraben near Troiseck. Pupa of the bark beetle *Ips cembrae* on *Larix decidua,* 01-VII-1995, *T. Kirisits PREM57490 (culture CMW10564).* Herbarium specimens of holotype and paratype have been deposited in the National Collection of Fungi (PREM), Pretoria, South Africa.

DISCUSSION

In this study we have shown that a collection of *Ophiostoma* isolates from Austria and Azerbaijan, peripherally resembling *O. stenoceras* and *O. nigrocarpum,* represent two new taxa. These first were recognized based on DNA sequence comparisons, but they could be distinguished subsequently using morphological characteristics. The recognition of *O. fusiforme* and *O. lunatum* as new species confirms previous views that what has been referred to as *O. stenoceras* and *O. nigrocarpum* represent complexes of several cryptic species (Pipe et al 2000, de Beer et al 2003). An interesting outcome of this study is that two authentic isolates of *O. nigrocarpum,* including the ex-type, did not group in what has been referred to previously as the *O. nigrocarpum* complex (de Beer et al 2003). This, as well as clear morphological and cultural differences, suggests that *O. nigrocarpum,* represented in this study by the two authentic strains of Davidson, is more distantly related to *O. stenoceras* and *S. schenckii* than previously realized.

The teleomorph of *O. fusiforme* resembles that of *O. stenoceras,* although the descriptions of *O. stenoceras* vary (Robak 1932, Davidson 1942, Aoshima 1965, Mariat 1971, Upadhyay 1981, Kowalski and Butin 1989). The ascospores produced by the ex-type culture of *O. stenoceras* are shorter than those of *O. fusiforme.* The new species also differs from *O. stenoceras* in culture morphology and the size and shape of its conidia. For example *O. fusiforme* gives rise to floccose cultures, different to the sparse and flat mycelium of *O. stenoceras.* Conidia in *O. fusiforme* are mostly fusiform and easily distinguished from the broadly ellipsoidal conidia of *O. stenoceras.* The anamorph of *O. fusiforme* is similar to *Sporothrix inflata* de Hoog (de Hoog 1974), which has conidiophores scattered, arising orthotropically or plagiotropically from undifferentiated hyphae, often in a terminal position or integrated in short straight or curved side branches. However, *S. inflata* is gray to olivaceous in culture, which is different from the white cultures of *O. fusiforme.*

The teleomorph of *O. lunatum* shares some similarity with *O. stenoceras* and *O. fusiforme.* The most important character distinguishing this species from the others is the conidia that are flattened at one side or curved with a blunt base. This is different from *O. fusiforme,* which has fusiform conidia, and *O. stenoceras,* with ellipsoidal conidia. Conidiogenous cells of *O. lunatum* arising orthotropically from undifferentiated hyphae sometimes are integrated in short branches. The anamorph stage of *O. lunatum* is reminiscent of *Sporothrix curviconia* de Hoog. However, the conidia of *O. curviconia* are substantially larger than those of *S. lunatum* (de Hoog 1974).

The three *Sporothrix* isolates from Canada and the U.S.A. were different morphologically from the anamorphs of *Ophiostoma fusiforme, O. lunatum* and *O. stenoceras.* Conidia in *O. fusiforme* are guttuliform to fusiform, those in *O. lunatum* are clavate or crescent shaped, and conidia of *O. stenoceras* are broadly ellip-

soidal. This is in contrast to conidia in the three *Sporothrix* isolates from Canada, which were cylindrical. De Beer et al (2003) included the *Sporothrix* isolate from Canada that also was used in this study. They assigned this isolate to the *O. nigrocarpum*-complex. Comparisons of our sequence data, however, show that the two authentic *O. nigrocarpum* isolates are distinct from all other species in our study. The colony morphology of the three *Sporothrix* isolates from Canada and the U.S.A. was different from that of the *O. nigrocarpum* isolates. Colonies of *O. nigrocarpum* were brownish-gray to blackish after perithecia formation, while colonies of these three isolates were white. The conidiogenous cells and conidia are similar in shape, but those of *O. nigrocarpum* are smaller and wider than those of the three *Sporothrix* isolates. Conidia of *O. nigrocarpum* also are rounded basally with inconspicuous scars, but in these isolates they are pointed. Our results suggest that the group that de Beer et al (2003) referred to as the *O. nigrocarpum* complex is not that species but an undescribed taxon (third subclade, Clade I). Because the teleomorph could not be obtained, we have chosen not to provide a name for this fungus but rather to treat it as an undescribed *Sporothrix* sp.

ACKNOWLEDGMENTS

We thank Renate Zipfel for providing us with a new and previously unpublished DNA extraction technique, Irene Barnes for advice regarding DNA sequences, Dr Erhard Halmschlager (IFFF-BOKU) and other collectors for supplying cultures and Dr Hugh Glen for providing the Latin diagnoses. We also acknowledge members of the Tree Protection Co-operative Programme (TPCP), the National Research Foundation (NRF), the THRIP initiative of the Department of Trade and Industry, South Africa, for financial assistance, and the University of Pretoria for a postdoctoral fellowship to Dilzara N. Aghayeva.

LITERATURE CITED

Aoshima K. 1965. Studies on wood-staining fungi of Japan [Doctoral Dissertation]. Tokyo, Japan: Tokyo University (English summary). 22 p.

Benade E, Wingfield MJ, Van Wyk PS. 1995. Conidium development in the *Hyalorhinocladiella* anamorph of *Ophiostoma ips*. Mycologia 87:298–303.

———, ———, ———. 1996. Conidium development in the *Hyalorhinocladiella* anamorph of *Ceratocystiopsis minuta-bicolor* and *Ophiostoma minus*. Can J Bot 74: 891–897.

———, ———, ———. 1997. Conidium development in *Sporothrix* anamorphs of *Ophiostoma*. Mycol Res 101: 1108–1112.

Brasier CM. 1981. Laboratory investigation of *Ceratocystis ulmi*. In: Stipes RJ, Campana RJ, eds. Compendium of elm diseases. St. Paul Minnesota: American Phytopathological Society. p 76–79.

———. 2000. Intercontinental spread and continuing evolution of the Dutch elm disease pathogens. In: Dunn CP, ed. The elms: breeding, conservation and disease management. Boston, USA, Dordrecht, The Netherlands, London, UK: Kluwer Academic Publishers. p 61–72.

Davidson RW. 1942. Some additional species of *Ceratostomella* in the United States. Mycologia 34:650–662.

———. 1966. New species of *Ceratocystis* from conifers. Mycopath Mycol Appl 28:273–286.

de Beer ZW, Harrington TC, Vismer HF, 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. St. Paul, Minnesota: American Phytopathological Society. p. 53–60.

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.

Hunt J. 1956. Taxonomy of the genus *Ceratocystis*. Lloydia 19:1–58.

Jacobs K, Wingfield MJ. 2001. *Leptographium* species. Tree pathogens, insect associates and agents of blue stain. St. Paul, Minnesota: American Phytopathological Society. 207 p.

Kowalski T, Butin H. 1989. Taxonomie bekannter und neuer *Ceratocystis*-Arten an Eiche (*Quercus robur* L.). J Phytopathol 124:236–248.

Mariat F. 1971. Adaptation de *Ceratocystis* a la vie parasitaire chez l'animal—etude de l'aquisition d'un pouvoir pathogene comparable a celui de *Sporothrix schenckii*. Sabouraud 9:191–205.

Middelhoven WJ, Guého E, de Hoog GS. 2000. Phylogenetic position and physiology of *Cerinosterus cyanescens*. Anton Leeuw 77:313–320.

Mouton M, Wingfield MJ, van Wyk PS. 1992. The anamorph of *Ophiostoma francke-grosmanniae* is a *Leptographium*. Mycologia 84:857–862.

Okada G, Seifert KA, Takematsu A, Yamaoka Y, Miyazaki S, Tubaki K. 1998. A molecular phylogenetic reappraisal of the *Graphium* complex based on 18S rDNA sequences. Can J Bot 74:1495–1506.

Pipe ND, Brasier CM, Buck KW. 2000. Evolutionary relationships of the Dutch elm disease fungus *Ophiostoma novo-ulmi* to other *Ophiostoma* species investigated by restriction fragment length polymorphism analysis of the rDNA region. J Phytopathol 148:533–539.

Rayner RW. 1970. A mycological colour chart. Kew, Surrey: Commonwealth Mycological Institute and British Mycological Society. 34 p.

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

Robak H. 1932. Investigations regarding fungi on Norwegian ground wood pulp and fungal infection at wood pulp mills. Nyt Mag f Naturv LXXI:185–330.

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. St. Paul, Minnesota: American Phytopathological Society. p 141–151.

———, Okada G. 1993. *Graphium* anamorphs of *Ophiostoma* species and similar anamorphs of other Ascomycetes. In: Wingfield MJ, Seifert KA, Webber JF, eds. *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. St. Paul, Minnesota: American Phytopathological Society. p 27–42.

Swofford DL. 1998. PAUP: Phylogenetic Analysis Using Parsimony. Version 4. Sunderland, Massachusetts: Sinauer Associates.

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

———. 1993. Classification of the Ophiostomatoid fungi. In: Wingfield MJ, Seifert KA, Webber JF, eds. *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. St. Paul, Minnesota: American Phytopathological Society. p 7–14.

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

Wingfield MJ, Seifert KA, Webber JF. 1993. *Ceratocystis* and *Ophiostoma*: taxonomy, ecology and pathogenicity. St. Paul, Minnesota: American Phytopathological Society. 293 p.