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Characterisation of *Ophiostoma* species associated with pine bark beetles from Mexico, including *O. pulvinisporum* sp. nov.

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Bark beetles (*Coleoptera: Scolytidae*) are common vectors of *Ophiostoma* species. These fungi include primary tree pathogens and important sapstain agents. In Mexico, *Ips calligraphus* and *Dendroctonus mexicanus* occur on many species of pine. *Pinus maximinoi* and *P. pseudostrobus* are the hosts of both species of insects. Little research has been done on ophiostomatoid fungi associated with pine bark beetles in Mexico. We recently obtained specimens of these bark beetles and their galleries from Mexico. The aim of the study was to isolate and identify *Ophiostoma* species associated with the two beetle species. In total, six ophiostomatoid species were found to be associated with them. These included *Ceratocystiopsis minuta*, *Ophiostoma pluriannulatum*, an *O. galeiformis*-like species, two unidentified *Sporothrix* spp., as well as a new species similar to *O. adjuncti*, *O. ips*, and *O. montium*, that we name as *O. pulvinisporum* sp. nov.

INTRODUCTION

Pinus species are native to the Northern Hemisphere, and the genus is one of the largest groups of conifers (Richardson 1998). Pine trees usually comprise a significant component of the ecosystems where they grow. The greatest number of *Pinus* species occurs in North and central American countries such as Mexico (Price, Liston & Struss 1998). Many bark beetle species (*Coleoptera: Scolytidae*) infest pines. In Mexico, two bark beetle species, *Ips calligraphus* and *Dendroctonus mexicanus*, occur on indigenous *Pinus* species (Wood & Bright 1992). *D. mexicanus* is known to infest and kill 21 species of pines, of which *Pinus pseudostrobus* is one of the most important (Marmolejo-Moncivais 1989, Marmolejo & García-Ocañas 1993). *Ips calligraphus* infests six species of pine, in some cases as a primary insect but in others it is secondary, with *P. maximinoi* as one of its most common hosts in tropical environments.

Many bark beetle species act as vectors of fungi, particularly ophiostomatoid fungi (Münch 1907, Whitney 1982, Beaver 1989, Paine, Raffa & Harrington 1997). Some ophiostomatoid fungi are primary pathogens (Harrington 1988, Brasier & Mehrotra 1995), and many are the causal agents of sapstain (Lagerberg, Lundberg & Melin 1927, Seifert 1993). At least 15 ophiostomatoid species have been reported from Mexico (Table 1). Recently, we had the opportunity to examine bark beetles and their galleries from Mexico and isolated ophiostomatoid fungi from the beetles and their galleries. The aim of this study was to identify the *Ophiostoma* and allied species associated with two beetle species. Light microscopy and sequences of the ITS region of the rRNA operon were employed to identify the isolates.

MATERIALS AND METHODS

Isolation of fungi

Fungi were isolated from bark beetles as well as from their galleries collected from Chiapas, Mexico. 35 galleries of *Dendroctonus mexicanus* infesting dying *Pinus pseudostrobus* and 20 of *Ips calligraphus* infesting dying *P. maximinoi* were collected.

Galleries were carefully examined using a dissection microscope. Spore masses accumulating at the tips of perithecia or conidiophores were carefully lifted using

Table 1. Ophiostomatoid fungi reported from Mexico.

Fungal species	Host	Insect	Sources[a]
Ophiostoma abietinum	*Abies vejari*	*Pseudohylesinus* species	1, 2, 3
O. conicolum	*Pinus cembroides*	*Conophthorus cembroides*	1, 2, 3
O. hyalothecium	*P. pseudostrobus*		1
O. ips	*P. teocote*, *P. pseudostrobus*	*Dendroctonus mexicanus*; *Ips* species	1, 3
O. minus	*P. arizonica*		3
O. piceae	*Quercus affinis*		3
O. piliferum	*P. hartwegii*		3
O. pluriannulatum	*Q. affinis*, *P. pseudostrobus*		3
Ceratocystis tubicollis[b]	*P. teocote*	*D. valens*	1
C. adiposa	Soil		4
C. fimbriata	*Hevea brasiliensis*		5
Ceratocystiopsis collifera	*P. teocote*, *P. hartwegii*	*D. valens*	1, 2, 3
C. fasciata	*P. pseudostrobus*	*D. mexicanus*	1, 3
C. minuta	*P. pseudostrobus*	*D. mexicanus*	1, 3
Sporothrix schenckii	Human		6

[a] 1, Marmolejo-Moncivais (1989); 2, Marmolejo & Butin (1990); 3, Marmolejo & García-Ocañas (1993); 4, Reyes & Castillo (1981); 5, Martin (1947); 6, Travassos & Lloyd (1980).
[b] Should be transferred to *Ophiostoma* according to Wingfield, Seifert & Webber (1993).

a fine sterile needle and transferred to a medium selective for *Ophiostoma* species (20 g Biolab malt extract, 20 g Biolab agar and 1000 ml deionised water, amended with 0.05% cycloheximide and 0.04% streptomycin).

Beetles from the same galleries were squashed directly onto the selective medium in Petri dishes. Cultures were incubated at 25 °C in the dark and purified by transferring mycelium from the edges of single colonies to fresh 2% MEA (20 g Biolab malt extract, 20 g Biolab agar and 1000 ml deionised water). All cultures used in this study are maintained in the Culture Collection (CMW) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Morphological studies

Both teleomorph and anamorph fruiting structures, when present, were mounted in lactophenol cotton blue on glass slides and examined microscopically. Isolates with *Hyalorhinocladiella* anamorphs resembling the anamorph of *Ophiostoma adjuncti*, *O. ips*, and *O. montium* were also grown on 2% WA (20 g Biolab agar and 1000 ml deionised water) with sterilised pine twigs to induce production of perithecia. For isolates with *Hyalorhinocladiella* anamorphs, 50 measurements were made for each structure, and the ranges and averages were computed.

Growth studies

The optimal growth temperature for selected isolates (CMW9023, CMW9024, and CMW9028) resembling the anamorph of *Ophiostoma adjuncti*, *O. ips* and *O. montium*, was determined by growing them at temperatures ranging from 5–35 °, at 5 ° intervals. Each isolate was inoculated onto the agar surface in six 2% MEA plates for each temperature, with a 6.0 mm diam agar disk taken from the actively growing margin of a fresh isolate. Colony diameters were measured after 4 and 8 d, and an average was calculated from six random measurements. Growth rates of three *O. ips* isolates (CMW6445, CMW9013, and CMW9319) and one *O. adjuncti* isolate (CMW135) were determined in a similar way.

Mating experiments

Mating experiments were conducted with isolates (CMW9024 and CMW9028) from Mexico that resemble *Ophiostoma adjuncti*, *O. ips*, and *O. montium* to determine thallism and to obtain perithecia. Ten single ascospore cultures were prepared from perithecia obtained in a cross between the two isolates. The single ascospore cultures were crossed in every possible combination. To induce production of perithecia, these cultures were incubated at 25 ° in the dark on 2% WA with sterilised pine twigs for three weeks. Some crosses gave rise to sexual structures, and it was thus possible to select tester strains of opposite mating type. Ten single conidium cultures were also prepared from each of the two isolates, and the tester strains were then crossed with the single conidium cultures. The thallism of *O. ips* isolates (CMW6418 and CMW6463) was determined in a similar way, except that 30 single ascospore and 30 single conidium cultures were made for each isolate.

DNA sequencing and phylogenetic analysis

Some isolates reproduced only asexually in culture and were difficult to identify based on morphology. Of these, 11 single hyphal tip isolates resembling *Sporothrix* or *Hyalorhinocladiella* were selected for sequencing (Table 2). Some of these isolates resembled the

Table 2. Fungi isolated in this study from bark beetles and their galleries in Mexico.

Species	Isolation no.[a]	GenBank accession no.	Host	Insect
Ceratocystiopsis minuta	CMW10771		*Pinus pseudostrobus*	*Dendroctonus mexicanus*
Ophiostoma galeiformis-like	CMW9490		*P. pseudostrobus*	*D. mexicanus*
Sporothrix sp. 1	**CMW9485**	AY546718	*P. pseudostrobus*	*D. mexicanus*
	CMW9486	AY546719		
	CMW9488	AY546720		
	CMW9491	AY546721		
	CMW9492	AY546722	*P. maximinoi*	*Ips calligraphus*
Sporothrix sp. 2	**CMW9487**	AY546694	*P. pseudostrobus*	*D. mexicanus*
	CMW9489	AY546695		
O. pluriannulatum	CMW10772		*P. pseudostrobus*	*D. mexicanus*
	CMW10773		*P. maximinoi*	*I. calligraphus*
O. pulvinisporum	**CMW9020**	AY546713	*P. pseudostrobus*	*D. mexicanus*
	CMW9022	AY546714		
	CMW9023			
	CMW9024		*P. maximinoi*	*I. calligraphus*
	CMW9026	AY546715		
	CMW9028			
	CMW9493	AY546716		

[a] Isolate numbers in bold type refer to isolates used for rDNA sequence analyses.

anamorph and culture morphology of *Ophiostoma adjuncti*, *O. montium* and *O. ips*. For these species, only one reference sequence was available from GenBank for *O. ips*. One isolate of *O. adjuncti*, three of *O. montium*, and 14 of *O. ips* originating from other parts of the world, were therefore included in the study (Table 3). For isolates with *Sporothrix* anamorph resembling *O. abietinum*, *O. nigrocarpum*, and *O. stenoceras*, reference sequences (Table 3) were obtained from GenBank mainly from the studies of De Beer *et al.* (2003) and Aghayeva *et al.* (2004).

DNA was extracted using a modified version of the extraction method developed by Raeder & Broda (1985). The ITS1 and ITS2 (internal transcribed spacer) regions, including the 5.8S gene of the ribosomal RNA operon, were amplified using primers ITS1-F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990). PCR products were sequenced with the same primers used for PCR, and two additional internal primers, CS2 (Wingfield *et al.* 1996) and ITS3 (White *et al.* 1990). Conditions for PCR amplification and sequencing reactions were as described by Zhou *et al.* (2004).

The resulting sequences were first aligned using ClustalX (1.81) and further manually aligned using Sequence Navigator Version 1.01 (ABI PRISM, PerkinElmer). Phylogenetic relationships among the isolates were determined using distance analyses in PAUP* Version 4 (Swofford 1998). Trees were constructed using the neigbour-joining tree building algorithm (Saitou & Nei 1987). The trees were rooted using GenBank sequences of *O. quercus* (AF198238 and AF493239). Bootstrap analysis (1000 replicates) was run to determine confidence levels of the branching points.

RESULTS

Isolation of fungi

We obtained 25 fungal isolates from the specimens collected. Of these, 16 isolates were from *Dendroctonus mexicanus* and nine from *Ips calligraphus*. Eighteen isolates, representing all the morphological groups present, were selected for further investigation (Table 2).

Morphological studies

Morphological study of isolates indicated that six ophiostomatoid species, *Ceratocystiopsis minuta*, an *Ophiostoma galeiformis*-like species, *O. pluriannulatum*, a *Hyalorhinocladiella* species, and two *Sporothrix* species, were associated with *Dendroctonus mexicanus*. Three species, *O. pluriannulatum*, a *Sporothrix* species and a *Hyalorhinocladiella* species, were collected from the galleries of *Ips calligraphus*.

Growth studies

The isolates with *Hyalorhinocladiella* anamorph from Mexico grew optimally at 30 °, while strains of *Ophiostoma ips* grew best at 25 °, reaching 58 and 50 mm diam in 4 d, respectively. None of the isolates grew at 5 °, and minimal growth occurred at 10 and 35 °. The isolate of *O. adjuncti*, however grew best at 20 °, reaching 25 mm diam in 4 d, and no growth occurred at 5, 30 and 35 °.

Mating experiments

None of the ten single ascospore or 20 single conidium cultures of the isolates with *Hyalorhinocladiella*

Table 3. Isolates of selected species of *Ophiostoma* used for comparative purpose in this study.

Species (previous name)	Isolate[a]	Other no.	GenBank no.	Collector/supplier	Origin	Host/insect
O. abietinum		CBS125.89[b]	AF484453	JG Marmolejo	Mexico	*Abies vejari*
O. adjuncti	CMW135[b]	C569	AY546696	RW Davidson	USA	*Pinus ponderosa*/ *Dendroctonus ponderosae*
O. fusiforme	CMW9968[b]	CBS112912[c]	AY280481	DN Aghayeva	Azerbaijan	*Populus nigra*
	CMW7131	CBS112925	AY280497	E Halmschlager	Austria	*Quercus petraea*
O. ips		C327[d]	AF198244	TC Harrington	USA	*Pinus* sp./*Ips pini*
	CMW312		AY546699	P Bedker	USA	*P. resinosa*
	CMW1173		AY546700	Mendel	Israel	*Crypturgus mediteranous*
	CMW5089		AY546701	XD Zhou	Chile	*Pinus radiata*/ *Hylurgus ligniperda*
	CMW6402		AY546697	XD Zhou	Chile	*P. radiata*/*H. ligniperda*
	CMW6418		AY546702	XD Zhou	South Africa	*P. elliottii*/ *Orthotomicus erosus*
	CMW6463		AY546703	XD Zhou	South Africa	*P. elliottii*/*H. ligniperda*
	CMW6445			XD Zhou	South Africa	*P. patula*/*Hylastes angustatus*
	CMW7075[b]	CBS137.36	AY546704	CT Rumbold	USA	*I. integer*
	CMW7076	CBS151.54	AY546705	A Käärik	Sweden	*O. proximus*
	CMW7079	CBS438.94	AY546706	T Kirisits	Austria	*I. sexdentatus*
	CMW9005		AY546698	XD Zhou	Sweden	*P. sylvestris*/*I. acuminatus*
	CMW9013			XD Zhou	Sweden	*P. sylvestris*/*I. acuminatus*
	CMW9319			XD Zhou	France	*P. sylvestris*/*I. sexdentatus*
	CMW13217[e]	UAMH9962[f]	AY546707	J Reid	Canada	*P. resinosa*
	MW13218[e]	SYPT1	AY546708	SH Kim	USA	*P. palustris*
	CMW13219[e]	SYPT2	AY546709	SH Kim	USA	*P. palustris*
O. pulvinisporum (*O. ips*)	CMW13216[e]	ATCC24285[g]	AY546717	HS Whitney	Canada	*P. contorta*
O. lunatum	CMW10563[b]	CBS112927	AY280485	T Kirisits	Austria	*Carpinus betulus*
	CMW10564	CBS112928	AY280486	T Kirisits	Austria	*Larix decidua*
O. montium	CMW13220[e]	UAMH4838	AY546710	Y Hiratsuka	Canada	*P. contorta*
	CMW13221[e]	CBS151.78	AY546711	RW Davidson	USA	*P. ponderosa*/*D. ponderosae*
	CMW13222[e]	92-628/55/4	AY546712	H Solheim	Canada	*P. contorta*/*D. ponderosae*
O. nigrocarpum	CMW650[b]	CBS637.66	AY280489	RW Davidson	USA	*Abies* sp.
	CMW651	CBS638.66	AY280490	RW Davidson	USA	*Pseudotsuga menziesii*
O. quercus	CMW7650	CBS102352	AF198238	PT Scard; JF Webber	UK	*Quercus* sp.
	CMW2463		AF493239	M Morelet	France	*Fagus sylvatica*
O. stenoceras	CMW3202[b]	CBS237.32	AF484462	H Robak	Norway	Pine pulp
	CMW129	C80	AF484456	RW Davidson	USA	
Sporothrix schenckii	CMW7611	MRC6856[h]	AF484469	HF Vismer	South Africa	Human sporotrichosis
	CMW7613	MRC6864	AF484470	HF Vismer	South Africa	Human sporotrichosis

[a] CMW = Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
[b] Ex-type culture.
[c] CBS = Culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, Netherlands.
[d] C = Culture collection of T. C. Harrington, Department of Plant Pathology, Iowa State University, USA.
[e] Isolates provided by Dr. Jae-Jin Kim.
[f] UAMH = University of Alberta Microfungus Collection.
[g] ATCC = American Type Culture Collection.
[h] MRC = Culture collection of PROMEC, Medical Research Council, Cape Town, South Africa.

anamorph produced perithecia. However, when paired with each other, 30 of the 45 pairs gave rise to perithecia. 20 of 30 single ascospore cultures, and 24 of 30 single conidium cultures of *Ophiostoma ips* isolates produced perithecia.

Sequence analysis

DNA fragments approximately 540 and 590 bp in size were amplified from the DNA of isolates with *Sporothrix* and *Hyalorhinocladiella* anamorphs, respectively (Table 2). Alignment of these sequences resulted in a total of 683 characters that were used in a distance analysis. There were two main clades both with a bootstrap support of 98% in the phylogram (Fig. 1) obtained with the neigbour-joining tree-building algorithm. The first clade, including three subclades, represented *Ophiostoma montium*, *O. ips*, and an undescribed taxon with high bootstrap supports of 100, 91, and 100%, respectively. The isolate of *O. adjuncti* (CMW135) grouped with *O. ips* with a bootstrap support of 64%. The second main clade included seven sub-clades with significant ($\geqslant$75%) bootstrap support at the terminal nodes. These represented *O. nigrocarpum*, *O. fusiforme*, *O. lunatum*, *O. stenoceras*, and *Sporothrix schenckii*. The isolate of *O. abietinum*

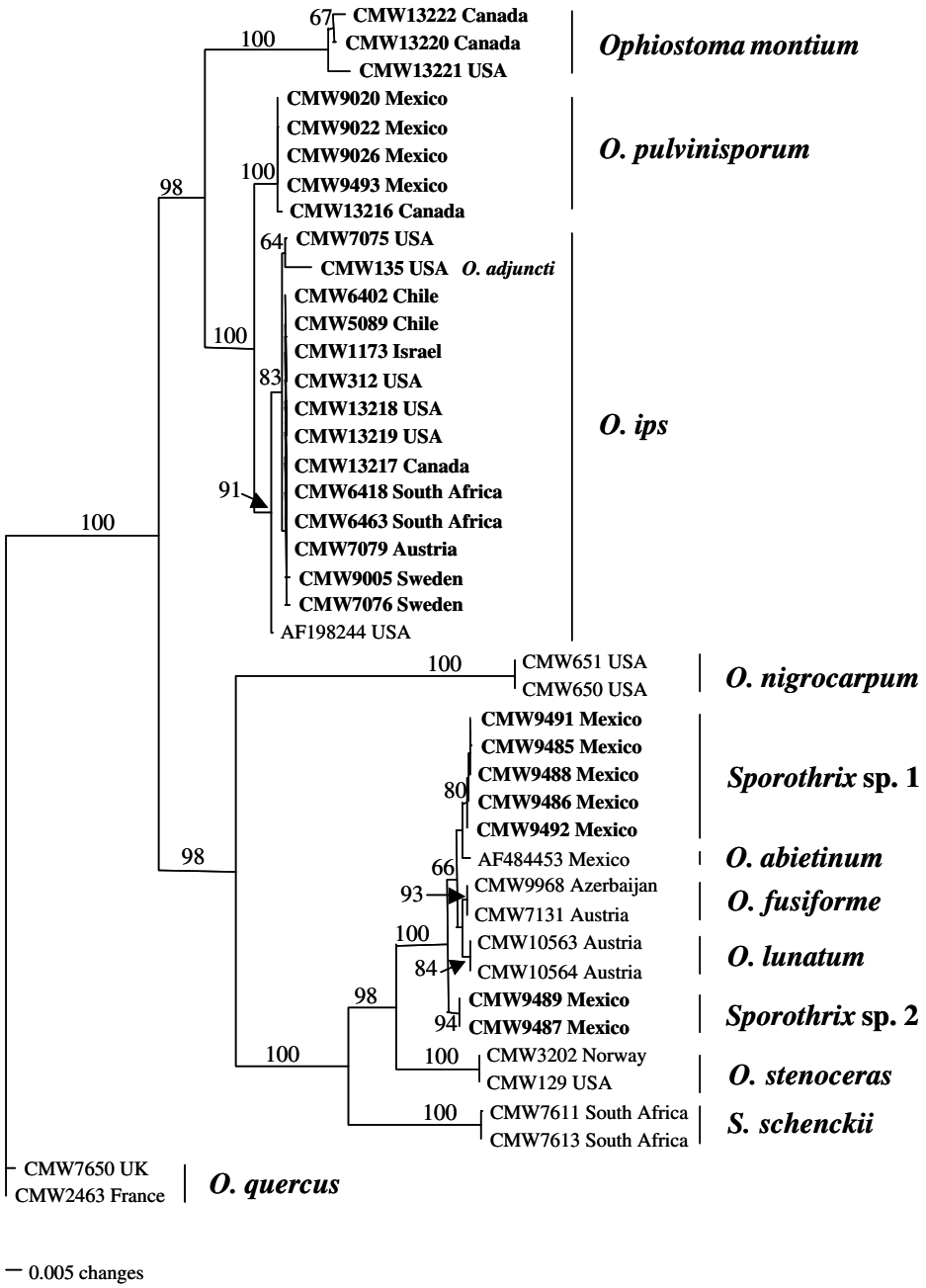

Fig. 1. Phylogram of the *Ophiostoma* species based on analyses of ITS sequences (ITS1 and ITS2 regions, including 5.8S rRNA gene) generated with the neigbour-joining tree building algorithm in PAUP*. Numbers of isolates sequenced in this study are printed in bold. *Ophiostoma quercus* was used as the outgroup taxon. Bar = total nucleotide differences between taxa. Bootstrap values (%) are indicated above the branches.

did not group within any of the sub-clades. The seven isolates with *Sporothrix* anamorph obtained in the study formed two distinct sub-clades (*Sporothrix* sp. 1 and *Sporothrix* sp. 2), with bootstrap supports of 80 and 94%, respectively.

TAXONOMY

Based on ITS sequence comparisons, mating reactions, growth studies, and morphology, we conclude that the fungus from Mexico that superficially resembles *O. adjuncti*, *O. ips*, and *O. montium*, represents a distinct taxon. This is described as follows:

Ophiostoma pulvinisporum X. D. Zhou & M. J. Wingfield, **sp. nov.** (Fig. 2A–G)

Etym.: From the Latin *pulvinus* (cushion) and *sporus* (spore); the epithet refers to the pulvinate ascospores of this species.

Perithecia in su perficie 2% WA efficientur ubi proles aliae aluntur. Bases peritheciorum, (150–) 233–319 (–400) μm diametro, hyphis aseptatis laete griseis ornatae; colla,

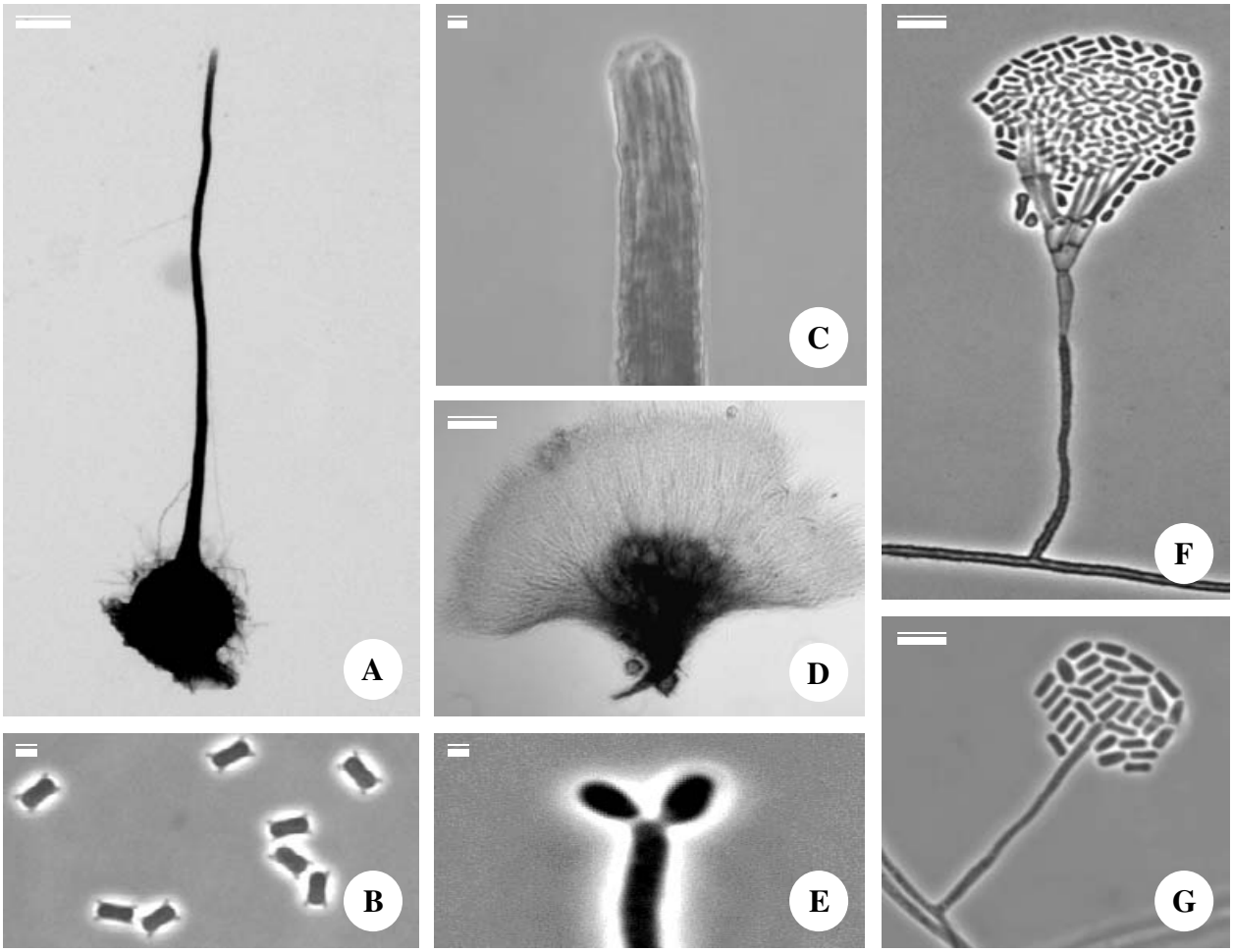

Fig. 2. *Ophiostoma pulvinisporum* (CMW9022) on 2% MEA. (A) Dark perithecia with long neck. Bar = 210 μm. (B) Pillow shaped ascospores. Bar = 3 μm. (C) Apex of the neck without ostiolar hyphae. Bar = 8 μm. (D) *Pesotum* anamorph. Bar = 11 μm. (E) Conidia of *Hyalorhinocladiella* anamorph. Bar = 2 μm. (F) *Leptographium* anamorph. Bar = 17 μm. (G) *Hyalorhinocladiella* anamorph. Bar = 9 μm.

(400–) 888–2152 (–3520) μm longa, basin versus (28–) 41–59 (–80) μm, apicem versus (10–) 11–23 (–48) μm lata. Hyphae ostiolares desunt. Ascosporae hyalinae, aseptatae, vaginatae, a latere fronteque visae pulviniformes, (3–) 3–5 (–6) × (1–) 1.5–2.5 (–3) μm, ab extremo visae quadrangulares.

Coloniae crescunt optime ad 30 ° in 2% MEA, 58 mm diametro quattuor diebus attingentes; laete griseae vel aetate atrobrunneae. Ad 5 ° non crescunt, et ad 10 et 35 ° minime crescunt.

Typus: **Mexico**: Chiapas, isol. ex *Dendroctonus mexicanus* infesting *Pinus pseudostrobus*, Feb. 2001, *M. J. Wingfield* (PREM57494 – holotypus; CMW9022 cultura viva).

Anamorphs: *Pesotum* (Fig. 2D), *Leptographium* (Fig. 2F), and *Hyalorhinocladiella* (Fig. 2E, 2G).

Perithecia produced superficially on 2% WA when strains of opposite mating type are crossed. Perithecial bases globose, dark, (150–) 233–319 (–400) μm diam (Fig. 2A), ornamented with aseptate light grey hyphae, (75–) 75–230 (–275) μm long, (1.5–) 1.5–2.5 (–2.5) μm wide. Perithecial necks dark brown to black, smooth, (400–) 888–2152 (–3520) μm long, (28–) 41–59 (–80) μm wide at base, (10–) 11–23 (–48) μm wide at the apex (Figs 2A, C). Ostiolar hyphae absent. *Asci* not observed. *Ascospores* hyaline, aseptate, with sheaths, pillow shaped in side and face view, (3–) 3–5 (–6) × (1–) 1.5–2.5 (–3) μm (Fig. 2B), quadrangular in end view.

Hyalorhinocladiella anamorph predominant, conidiophores: (70) 95–225 (–230) μm long; conidiogenous cells, (7.5–) 12–38 (–50) × (1.5–) 1.5–2.5 (–2.5) μm; conidia hyaline, ellipsoid to ovoid, (3–) 3.5–8.5 (–21) × (1–) 1.5–2.5 (–4) μm. *Leptographium* anamorph: conidiophores (60) 80–160 (–170) μm long; conidiogenous cells, (8.5–) 9.5–18.5 (–20.5) × (1.5–) 1.5–2.5 (–3) μm; conidia hyaline, oblong to ellipsoid with truncate bases, (2–) 2.5–5.5 (–6) × (1–) 1–2 (–2.5) μm. *Pesotum* anamorph: conidiophores (240)–260–340 (–360) μm long; conidiogenous cells, (20–) 25–35 (–45) × (1–) 1–1.5 (–2) μm; conidia hyaline, rod-shaped, (3–) 3–5 (–7) × (1–) 1–2 (–2.5) μm.

Colonies with optimal growth at 30 ° on 2% MEA, reaching 58 mm diam in 4 d. Colonies light grey (19″d) to dark mouse grey (13‴″k) with age (Rayner 1970). *Hyalorhinocladiella* anamorph dominant in cultures. Aerial mycelia extensively present. No growth at 5 °, and minimal growth at 10 and 35 °.

Additional specimens examined: **Mexico**: Chiapas, isolated from *Dendroctonus mexicanus* infesting *Pinus pseudostrobus*, Feb. 2001, *M. J. Wingfield* (CMW9020, PREM57495); *loc. cit*., isolated from *I. calligraphus* infesting *P. maximinoi*, Feb. 2001, *M. J. Wingfield* (CMW9026, PREM57496; CMW9493, PREM57497).

DISCUSSION

In this study, six ophiostomatoid species were found associated with *Dendroctonus mexicanus* and *Ips calligraphus* from Mexico. These included *Ceratocystiopsis minuta*, *Ophiostoma pluriannulatum*, an *O. galeiformis*-like species, two unidentified *Sporothrix* spp., as well as the new species similar to *O. adjuncti*, *O. ips*, and *O. montium*, named here as *O. pulvinisporum*.

C. minuta was first described by Siemaszko (1939) from *Picea abies* infested by *I. typographus* in Poland. This species is morphologically similar to *C. collifera* and *C. brevicomi* but the latter two species have typical collar-like structures (Marmolejo & Butin 1990, Hsiau & Harrington 1997), absent in *C. minuta*. *C. minuta* is commonly associated with *I. typographus* infesting Norway spruce, *Tomicus* species infesting *Pinus* species in Europe (Mathiesen-Käärik 1953, Solheim 1986), as well as with various conifer-infesting *Dendroctonus* and *Ips* species in North America, Australia, and Japan (Davidson 1942, Upadhyay 1981, Stone & Simpson 1990, Yamaoka *et al*. 1998). In South Africa, the fungus has been found on the exotic *Hylastes angustatus* and *Hylurgus ligniperda*, and it was evidently introduced into the country from Europe (Zhou *et al*. 2001). The presence of the fungus in Mexico is not surprising given its wide distribution in the Northern Hemisphere.

O. pluriannulatum was first described by Hedgcock (1906) from *Quercus borealis* in the USA. The fungus is known as a sapstain agent, especially of hardwoods, and as a fungal associate of many insects in the Northern Hemisphere (Lagerberg, Lundberg & Melin 1927, Hedgcock 1933, Hunt 1956), including Mexico, where it has been found on both *Quercus* and *Pinus pseudostrobus* (Marmolejo & García-Ocañas 1993). In the Southern Hemisphere, the fungus occurs in New Zealand on *P. radiata* (Farrell *et al*. 1997), and in South Africa it is associated with three pine-infesting bark beetle species (Zhou *et al*. 2001). This study represents the first report of the fungus from *P. maximinoi*. It is also the first time that *O. pluriannulatum* has been associated with a *Dendroctonus* species, and although it has been found on *Ips typographus* in Sweden (Mathiesen-Käärik 1953), it has not previously been reported from *I. calligraphus*.

O. galeiformis was first described by Bakshi (1951) from Scotland, and is associated with a wide variety of bark beetle species (Bakshi 1951, Mathiesen-Käärik 1953, Hunt 1956, Zhou *et al*. 2001). The isolate from Mexico (CMW9490) that we have tentatively assigned to this species closely resembles *O. galeiformis*, but the culture differs slightly from published descriptions. This fungus has not been reported from North America before, and it is possible that the isolate represents a distinct taxon. The taxonomy of this species is confused because the type material has apparently been lost. The identity of the isolate from Mexico will thus be considered in a future study focusing on isolates resembling this species from different parts of the world.

Ophiostoma abietinum, *O. nigrocarpum*, *O. fusiforme*, *O. lunatum*, and *O. stenoceras* are difficult to distinguish based on morphology alone. These species can, however, be distinguished from each other based on DNA sequences (De Beer *et al*. 2003, Aghayeva *et al*. 2004). Seven isolates with *Sporothrix* anamorph obtained from this study formed two distinct clades within this group. No sexual structures were observed for these fungi, and additional isolates, and possibly sequences of another gene are needed to clarify their identity.

O. pulvinisporum is morphologically similar to *O. adjuncti*, *O. ips*, and *O. montium*. These species are characterised by pillow-shaped ascospores with distinct sheaths. The anamorphs of the species form a continuum of conidiophore structures varying from single mononematous structures terminating in penicillately branched apices similar to *Leptographium*, to synnematous structures reminiscent of *Pesotum*. Anamorphs of *O. adjuncti*, *O. ips*, and *O. montium* have in the past been referred to the genera *Hyalorhinocladiella*, *Graphium* (now *Pesotum*), and *Leptographium*, because no single genus can accommodate the variety of structures produced by these species (Rumbold 1931, 1941, Hunt 1956, Davidson 1978, Upadhyay 1981, Wingfield, Seifert & Webber 1993, Okada *et al*. 1998). Harrington *et al*. (2001), however, restricted *Pesotum* to those anamorphs with affinities to the *O. piceae* complex. This implies that the synnematous anamorph of *O. ips* and related taxa should not be assigned to *Pesotum*. In our opinion, the *Hyalorhinocladiella* form of *O. pulvinisporum* is predominant, and if an anamorph genus were required, we would preferentially refer to it as *Hyalorhinocladiella*.

O. pulvinisporum and *O. ips* can be distinguished from each other based on their different growth rates, mating systems, and ITS rDNA sequences. *Ophiostoma pulvinisporum* grows optimally at 30 °, *O. ips* at 25 °, and *O. adjuncti* at 20 °. Kim *et al*. (2003) reported that *O. montium* was not able to grow at 35 ° while *O. pulvinisporum* and *O. ips* grew at this temperature. Our results also showed that *O. pulvinisporum* is heterothallic. In contrast, *O. ips* is homothallic. ITS rDNA sequence data comparisons in this study strongly supported the separation of *O. pulvinisporum*, *O. ips*, and *O. montium*. *Ophiostoma adjuncti* (CMW135) grouped with *O. ips*, although the bootstrap support was low (64%). Additional isolates of *O. adjuncti* are necessary to determine its relationship with *O. ips*.

O. pulvinisporum was isolated from *D. mexicanus* and *I. calligraphus*, occurring on *P. pseudostrobus* and *P. maximinoi*, respectively, in Mexico. Previously, *O. ips* has been reported from *D. mexicanus* and an *Ips* species in Mexico, infesting *P. pseudostrobus* and *P. teocote* (Marmolejo-Moncivais 1989, Marmolejo & García-Ocañas 1993). These reports of *O. ips* from Mexico might have represented *O. pulvinisporum*.

The presence of six species of fungi on the limited collection of material obtained for this study indicates that a large diversity of ophiostomatoid species are probably associated with pine bark beetles in Mexico. This is to be expected because Mexico is rich in native pine species (Richardson 1998). Further studies with additional bark beetles and pine species from this country will most likely reveal many more undescribed ophiostomatoid species.

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