



Chapter 3

Ophiostoma spp. associated with two pine-infesting bark beetles from Chile

Bark beetles (Coleoptera: Scolytidae) are common vectors of *Ophiostoma* spp., which include primary tree pathogens as well as important sapstain agents. In Chile, *Hylurgus ligniperda* and *Hylastes ater*, which are native to Europe, commonly occur on the exotic *Pinus radiata*. Little research has been done on *Ophiostoma* spp. associated with bark beetles in Chile and especially those carried by introduced pine-infesting insects. We recently obtained specimens of these bark beetles and their galleries, and the aim of this study was to isolate and identify *Ophiostoma* spp. associated with the two beetle species. Identification was achieved using morphological characteristics and where appropriate, DNA sequencing. A total of five ophiostomatoid species, *Ceratocystiopsis minuta*, *O. galeiformis*, *O. huntii*, *O. ips*, and *O. quercus*, were found associated with the bark beetles, all of which are recorded from Chile for the first time.

Keywords: *Leptographium*, Ascomycetes, Scolytidae, *Hylurgus*, *Hylastes*.

INTRODUCTION

Pinus spp. are native to the Northern Hemisphere, where species diversity is most pronounced in Central America and Southeast Asia (Richardson, 1998). Many *Pinus* spp., however, have been introduced into Southern Hemisphere countries such as New Zealand, Australia, Chile and South Africa. In these countries, some pine species are grown in very large commercial plantations (Le Maitre, 1998; Richardson, 1998).

Many bark beetle species (Coleoptera: Scolytidae) infest *Pinus* spp. (Wood & Bright, 1992). Most of these bark beetles are not considered as pests in their native environment, but when introduced into new areas and particularly where uniform stands of *Pinus* spp. are planted, they can become problematic (Wingfield & Swart, 1994; Wingfield *et al.*, 2001). In Chile, *Hylurgus ligniperda* (Fabricius) and *Hylastes ater* (Paykull) are exotic pests of European origin which infest exotic *P. radiata* (Wood & Bright, 1992; Billings, 1993). Both of these insects can infest fresh stumps and slash shortly after trees are felled (Ciesla, 1988).

Many bark beetles are also vectors of ophiostomatoid fungi, which include a number of primary pathogens and sapstain agents (Whitney, 1982; Harrington, 1988; Seifert, 1993; Brasier & Mehrotra, 1995; Paine, Raffa & Harrington, 1997). In Chile, at least eight ophiostomatoid species have been reported from different hosts (Table 1). Little research has, however, been conducted on the fungal associates of pine-infesting bark beetles in this country.

In Chile, as is the case in South Africa, exotic pine plantations constitute a significant section of the forestry industry. Considerable research has been conducted on pine bark beetle-associated fungi in exotic pine plantations of South Africa in recent years (Wingfield & Swart, 1989; Zhou *et al.*, 2001, 2002). A comparison of the fungi associated with introduced bark beetles in Chile, with fungi from the same niche in South Africa, could provide insight into the spread of the bark beetles and their fungi south of the equator.

Recently, we have had the opportunity to examine bark beetles and their galleries from Chile, and to isolate *Ophiostoma* spp. occurring on the beetles and in their galleries. The aim of this study was to identify these fungi based on morphology and comparisons of ITS rDNA sequences.

MATERIALS AND METHODS

Isolation of fungi

In the Valdivia area of Chile, 34 specimens of *H. ater* were collected from roots of dying *P. radiata* trees, and 80 specimens of *H. ligniperda* were collected from felled trees of the same species in log stacks. Four fungal isolates were collected directly from the galleries of *H. ater*. Isolation of fungi from bark beetles and their galleries was conducted in a similar way to that described by Zhou *et al.* (2001). 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, examined microscopically, and characteristic structures measured. To induce the production of perithecia, isolates presenting only anamorphs were grown on 2 % WA (20 g Biolab agar and 1000 ml distilled water), to which sterilised pine twigs had been added.

DNA sequencing and phylogenetic analysis

Isolates used. Some isolates produced only a *Pesotum*-like anamorph in culture, resembling that of *O. piceae* (Münch) H. & P. Sydow, *O. quercus* (Georgévitch) Nannfeldt, and *O. floccosum*

Mathiesen. These isolates were difficult to identify based on morphology, and for two of them (CMW9480 and CMW9481), single hyphal tip cultures, were prepared for sequencing (Table 2).

DNA extraction. Each culture was grown in 50 ml of malt extract broth (20 g Biolab malt extract, and 1000 ml distilled water) at 25 °C in the dark for 10 days. Mycelium was then harvested by filtration (Whatman no. 1 filter paper) and freeze-dried.

DNA was extracted using a modified version of the extraction method developed by Raeder and Broda (1985). Freeze-dried mycelium was grounded to fine powder in liquid nitrogen. Approximately 0.5 ml of mycelial powder was suspended in 800 µl of extraction buffer (200 mM Tris-HCl pH 8.0, 150 mM NaCl, 25 mM EDTA pH 8.0, 0.5 % SDS). Phenol (500 µl) and 300 µl of chloroform were added to the suspension, and the mixture was vortexed, then centrifuged in a Beckman JA 25.50 rotor (12,000 rpm, 60 min, 4 °C). The upper aqueous layer was transferred to sterilized Eppendorf tubes. 200 µl of phenol and an equal volume of chloroform were added, vortexed, and then centrifuged for 5 minutes. The aqueous phase was transferred again, and the chloroform extraction (400 µl) was repeated once or twice until the interface was clear. Nucleic acid was then precipitated with 0.1 vol. of 3 M NaAc (pH 5.4) and 1 vol. of isopropanol. The nucleic acid was pelleted using centrifugation (12,000 rpm, 30 min, 4 °C), and the salt removed by washing with 70 % ethanol. The vacuum-dried pellet was resuspended in 50 µl of sterile water and 2 µl of RNAase (10 mg / ml, Roche Molecular Biochemicals) was added to digest any RNA. The reaction was incubated in a water bath overnight at 37 °C. Agarose (Promega, Madison, CT, USA) gel electrophoresis (1%) was used to determine the presence of the DNA. The DNA was visualized using Ethium bromide and UV light. The concentration of the DNA was determined using UV spectroscopy (Beckman Du Series 7500 Spectrophotometer).

PCR amplification. The ITS1 and ITS2 (internal transcribed spacer) regions, including the 5.8S gene of the ribosomal RNA operon, were amplified, using primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes & Bruns, 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). The template DNA was amplified in a 50 μ l PCR reaction volume, consisting of 0.5 μ l of DNA solution (100-200 ng μ l⁻¹), 0.5 μ l of Expand High Fidelity PCR System enzyme mix (1.7 U) (Roche Molecular Biochemicals, Alameda, CA), 5 μ l of Expand HF buffer (10x) without MgCl₂, 3 μ l of MgCl₂ (25 mM), and 1.5 μ l of each primer (10 mM). PCR reactions were performed on an Eppendorf Mastercycler® Personal (PerkinElmer, Germany). The PCR conditions were as follows: 95 °C for 2 min, followed by 40 cycles, where each cycle included 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C. A final elongation step was conducted for 8 min at 72 °C. A negative control, using water without DNA, was included with each PCR. PCR products were visualized on a 1 % agarose gel stained with ethidium bromide (10 mg ml⁻¹) under UV illumination. Amplification products were purified using the High Pure PCR Product Purification Kit (Boehringer, Mannheim, Germany).

DNA Sequencing. Sequencing reactions were carried out with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PerkinElmer Applied BioSystems) following the manufacturer's instructions. Sequencing was performed on an ABI PRISM 377 Autosequencer (PerkinElmer Applied BioSystems, Foster City, CA, USA). PCR products were sequenced with the same primers used for PCR, as well as two additional internal primers, CS2 (5'-CAATGTGCGTTCAAAGATTCG-3') (Wingfield *et al.*, 1996), and ITS3 (5'-GCATAGATGAAGAAGCAGC-3') (White *et al.*, 1990).

Phylogenetic analysis. The obtained sequences were aligned using Sequence Navigator version 1.01 (ABI PRISM, PerkinElmer). This alignment was checked manually, and compared with data

of related isolates from other studies, obtained from GenBank (Table 2). Aligned data were analysed using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1998). Uninformative characters were excluded from the analyses. The most parsimonious trees were produced using a heuristic search with TBR (Tree Bisection and Reconstruction) branch swapping. Bootstrap analysis (1000 replicates) was run to determine confidence intervals of the branching points.

RESULTS

Isolation of fungi

In total, 45 fungal isolates were obtained from the specimens collected. Of these, 30 were isolated from *H. ligniperda*, and 15 from *H. ater*. Eleven isolates, representing all the morphological groups present, were selected for further investigation (Table 2).

Morphological studies

Morphological study showed that two ophiostomatoid fungi, *Ceratocystiopsis minuta* (Siem.) Upadhyay & Kendrick and *Ophiostoma ips* (Rumbold) Nannfeldt, were commonly associated with *H. ligniperda*. Three ophiostomatoid fungi, *O. galeiformis* (Bakshi) Mathiesen-Käärik, *O. huntii* (Rob.-Jeffer.) de Hoog & R. J. Scheff., and a *Pesotum* sp. resembling the anamorphs of *O. piceae*, *O. quercus*, and *O. floccosum*, were found associated with *H. ater*.

Sequence analysis

DNA fragments (574 bp) were amplified for the isolates with *Pesotum* anamorphs. Manual alignment of these sequences resulted in a total of 579 characters. Of these, 45 were parsimony informative, 6 parsimony uninformative, and 528 were constant. Heuristic searches without using the outgroup taxon, resulted in one most parsimonious tree (CI = 1.000, RI = 1.000, HI = 0.000),

in which three main clades were well resolved (Fig. 1). Two isolates obtained in this study (CMW 9480 and CMW 9481) resided in the first clade representing *O. quercus* group. The second and third clade represented *O. piceae* and *O. floccosum* groups, respectively, with bootstrap supports of 90 % and 100%.

DISCUSSION

In this study, three ophiostomatoid species were found associated with each of the two bark beetle species from Chile. From *Hylurgus ligniperda*, *Ceratocystiopsis minuta*, *Ophiostoma galeiformis* and *O. ips* were isolated, while *O. galeiformis*, *O. huntii*, and *O. quercus* were found with *Hylastes ater*. *Ophiostoma galeiformis* was the only fungal species present on both bark beetle species. This study represents the first report of these five fungal species from Chile, and this list considerably increases the number of ophiostomatoid fungi known from the country.

Ceratocystiopsis minuta was first described by Siemaszko (1939) from *Picea abies* infested by *Ips typographus* L. in Poland. The fungus is associated with different conifer-infesting bark beetles from many parts of the world (Davidson, 1942; Mathiesen-Käärrik, 1953; Upadhyay, 1981; Solheim, 1986; Stone & Simpson, 1990; Yamaoka *et al.*, 1998). In South Africa, *Cop. minuta* has been found on the exotic *Hylastes angustatus* (Herbst) and *Hylurgus ligniperda* (Zhou *et al.*, 2001). The presence of *Cop. minuta* on *H. ligniperda* infesting *P. radiata* in Chile is not surprising, given its association with the European bark beetles in South Africa.

Ophiostoma galeiformis is associated with many different bark beetle species. The fungus was first described by Bakshi (1951) in Scotland, where it was isolated from *Larix kaempferi* infested by *Hylurgops palliatus* (Gyll.), *Dryocoetes autographus* (Ratzeburg), and *Trypodendron lineatum* (Olivier) (Bakshi, 1951). In Sweden, *O. galeiformis* has been isolated from *Picea*

infested with *Hylastes cunicularius* (Errichson) (Mathiesen-Käärrik, 1953), as well as from pine-infesting bark beetles (Hunt, 1956). The discovery of the fungus from Chile in the present study is not unusual as it has also been found associated with the exotic pine-infesting *H. ligniperda* in South Africa (Zhou *et al.*, 2001). The *Hylastes* vectors of these fungi, however, differ in the two areas, although both originate in Europe.

Ophiostoma huntii has been associated with several different bark beetle species on *Pinus* and *Picea* spp. (Jacobs & Wingfield, 2001). This species was originally isolated from pine infested with a *Dendroctonus* sp. in Canada (Robinson-Jeffrey & Grinchenko, 1964), and has been reported to be associated with *D. ponderosae* (Hopk.), *Ips pini* (Say), *Hylastes macer* (LeConte), and *Tomicus piniperda* (Linnaeus) in Europe (Jacobs *et al.*, 1998) and the USA (Davidson & Robinson-Jeffrey, 1965; Harrington, 1988; Gibbs & Inman, 1991; Wingfield & Gibbs, 1991). *Ophiostoma huntii* occurs also in Australia and New Zealand (Jacobs *et al.*, 1998), where it is associated with the European root-infesting bark beetle, *H. ater*. Its presence on *H. ater* in Chile suggests that this insect was introduced into Chile from New Zealand, Australia, or at least from a common source. Studies on populations of fungi such as *O. huntii* in Chile, Australia and New Zealand, might provide useful information on how the bark beetles and fungi have been distributed throughout the Southern Hemisphere.

Ophiostoma ips was first described from *Ips calligraphus* on *P. echinata*, *P. sylvestris*, and *P. rigida* in the USA (Rumbold, 1931), and has since been widely reported to be the associate of many conifer-infesting bark beetles in the Northern Hemisphere (Rumbold, 1931; Nisikado & Yamauti, 1933; Mathiesen-Käärrik, 1953; Hunt, 1956; Mathre, 1964; Upadhyay, 1981; Rane & Tattar, 1987; Lieutier *et al.*, 1991; Perry, 1991; Masuya *et al.*, 1999). In the Southern Hemisphere, it has been reported in Australia from galleries of *Ips grandicollis* (Eichhoff) on *P. taeda* (Vaartaja, 1966; Stone & Simpson, 1990), and from New Zealand on *P. elliotii* and *P. radiata* (Hutchison & Reid, 1988; Farrell *et al.*, 1997). The fungus has also been reported from

South Africa associated with *O. erosus*, *H. angustatus* and *H. ligniperda* occurring on *P. radiata*, *P. patula* and *P. elliottii* (Wingfield & Marasas, 1980; Zhou *et al.*, 2001). In this study, *O. ips* was isolated from *H. ligniperda* on *P. radiata* in Chile, which is similar to the situation in South Africa.

Ophiostoma quercus (De Beer *et al.*, 2003) occurs primarily on hardwoods, but occasionally also on conifers, while *O. piceae* occurs almost exclusively on conifers (Harrington *et al.*, 2001; De Beer, Wingfield & Wingfield, 2003). These two species are morphologically almost indistinguishable, but can be separated based on ITS rDNA sequence data (Harrington *et al.*, 2001; De Beer, Wingfield & Wingfield, 2003). DNA sequence comparisons in the present study have confirmed the presence of *O. quercus* in association with *H. ater* on *P. radiata* in Chile. In South Africa, *O. quercus*, but not *O. piceae*, has, however, been reported from various hardwoods, and those identifications were confirmed with ITS rDNA sequencing (De Beer, Wingfield & Wingfield, 2003). The results of the present study thus suggest that previous reports of *O. piceae* from *Nothofagus*, *Laurelia*, and *Pinus* spp. in Chile (Butin & Aquilar, 1984; Butin & Peredo, 1986; Billings, 1993), might have represented *O. quercus*, and not *O. piceae*.

As in Chile, *H. ligniperda* and *H. ater* occur in Australia and New Zealand (Swan, 1942; Anonymous, 1974), whereas only *H. ligniperda* has been reported from South Africa (Tribe, 1991). While both these bark beetle species were introduced from Europe to these Southern Hemisphere countries, the pine species planted there originate from North America. Unfortunately very little is known regarding the fungal associates of these bark beetle species in their native environments in the Northern Hemisphere. Only from Sweden five ophiostomatoid species have been recorded from *H. ater*: *O. ips*, *O. penicillatum* Grossman, *O. piceae*, *L. lundbergii* Lagerb. & Melin, *O. piliferum* [= *O. coeruleum*], and *Graphium areum* Hedgc. (Mathiesen, 1950; Mathiesen-Käärik, 1953). None of these species have, however, been found in association with *H. ater* in the Southern Hemisphere to date. It appears that *H. ater* forms associations with different fungal

species in different environments. This might indicate that the relationship between bark beetle vector and fungus in this case is coincidental, rather than specific.

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Table 1. Ophiostomatoid fungi previously reported from Chile.

Fungal species	Host	Reference(s)
<i>Ophiostoma nothofagi</i> (Butin) Rulamort	<i>Nothofagus dombeyi</i>	Butin & Aguilar, 1984.
<i>O. piceae</i> (Münch) H. & P. Sydow	<i>Nothofagus</i> spp.; <i>Pinus</i> spp.; <i>Laurelia</i> spp.	Butin & Aguilar, 1984; Butin & Peredo, 1986; Billings, 1993; Harrington <i>et al.</i> , 2001.
<i>O. piliferum</i> (Fries) H. & P. Sydow	<i>N. pumilio</i>	Butin & Aguilar, 1984.
<i>O. valdivianum</i> (Butin) Rulamort	<i>N. alpina</i> ; <i>N. dombeyi</i>	Butin & Aguilar, 1984.
<i>Pesotum</i> sp.	<i>P. radiata</i>	Peredo & Alonso, 1988.
<i>Sporothrix curviconia</i> de Hoog	<i>P. radiata</i>	Peredo & Alonso, 1988.
<i>Sporothrix schenckii</i> Hekt. & Perkins	Human	Travassos & Lloyd, 1980.
<i>Sporothrix</i> sp.	<i>P. radiata</i>	Peredo & Alonso, 1988.

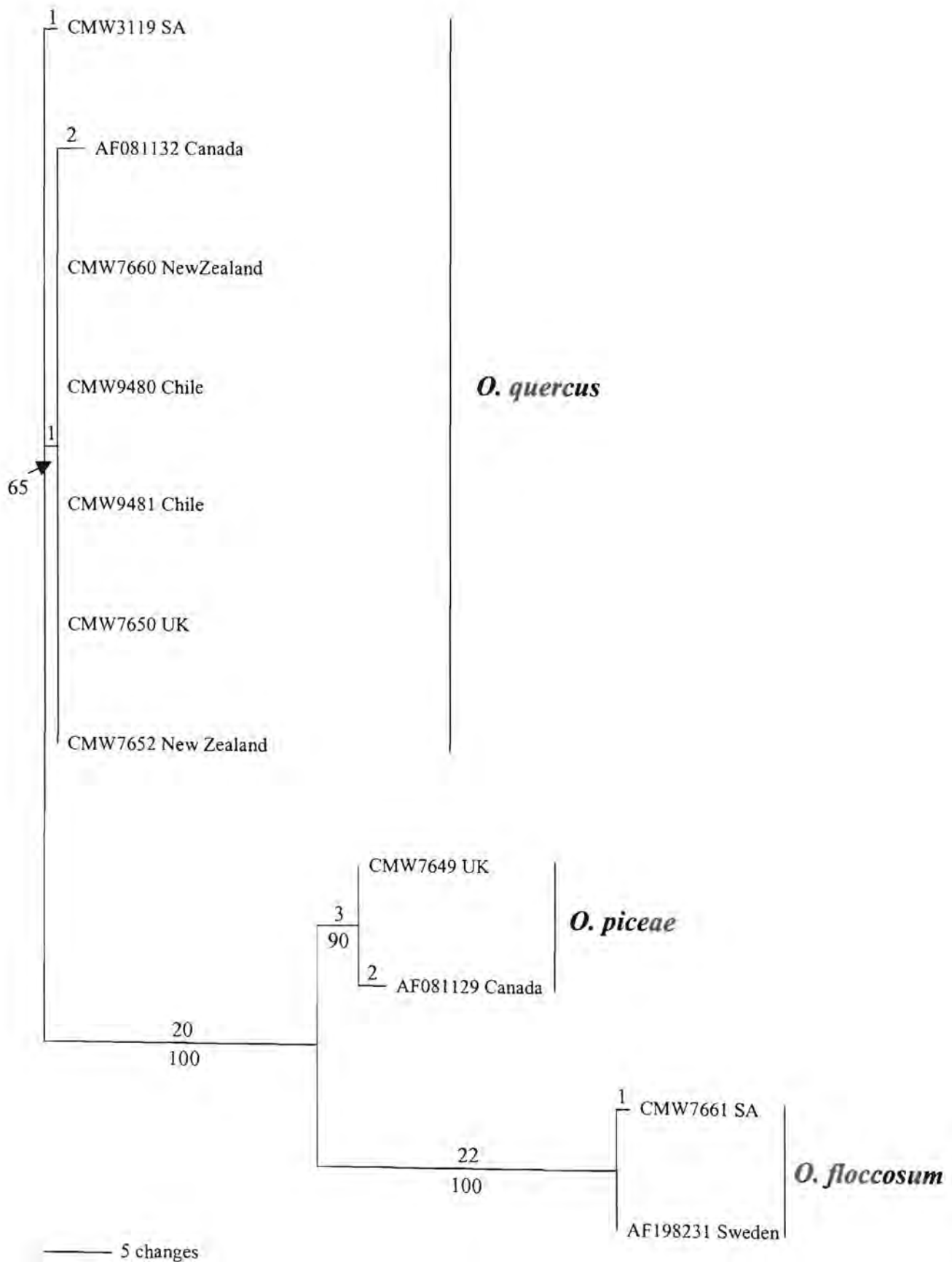
Table 2. Fungi isolated from bark beetles and their galleries in Chile and isolates of selected species used as reference material in this study.

Species	Isolate No.	GenBank No.	Collector/supplier	Origin	Host	Insect
<i>Ceratocystiopsis minuta</i>	^a CMW10770			Chile	<i>Pinus radiata</i>	<i>Hylurgus ligniperda</i>
<i>Ophiostoma galeiformis</i>	CMW9478, CMW9479			Chile	<i>P. radiata</i>	<i>Hylastes ater</i>
	CMW9482, CMW9483			Chile	<i>P. radiata</i>	<i>H. ligniperda</i>
<i>O. huntii</i>	CMW10768 CMW10769			Chile	<i>P. radiata</i>	<i>H. ater</i>
<i>O. ips</i>	CMW5089 CMW6402			Chile	<i>P. radiata</i>	<i>H. ligniperda</i>
<i>O. quercus</i>	^b CMW9480 ^b CMW9481			Chile	<i>P. radiata</i>	<i>H. ater</i>
<i>O. floccosum</i>	CMW7661	AF493253 AF198231	ZW de Beer A Kåårik	South Africa Sweden	<i>P. elliotii</i> <i>Picea</i> or <i>Pinus</i>	
<i>O. piceae</i>	CMW7649	AF081130 AF081129	JN Gibbs SH Kim <i>et al.</i>	UK Canada	<i>P. sylvestris</i> <i>Picea mariana</i>	
<i>O. quercus</i>	CMW7650 CMW3119 CMW7660 CMW7652	AF198238 AF081132 AF493244 AF493252	PT Scard, JF Webber SH Kim <i>et al.</i> ZW de Beer ZW de Beer RA Blanchette	UK Canada South Africa New Zealand New Zealand	<i>Quercus</i> sp. <i>Tsuga</i> <i>Pinus</i> chips <i>Pinus</i> chips <i>P. radiata</i>	

^a CMW = Culture Collection Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

^b Isolates used in rDNA sequence analyses.

Fig. 1. Phylogram of *Pesotum* group based on analyses of ITS sequences (ITS1 and ITS2 regions, as well as 5.8S rRNA gene). *Ophiostoma ips* was used as an outgroup. Base substitution numbers are indicated above the branches and the bootstrap values (1000 bootstrap repeats) below the branches.



Chapter 4

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

Bark beetles (Coleoptera: Scolytidae) are common vectors of *Ophiostoma* spp. These fungi include primary tree pathogens and important sapstain agents. In Mexico, *Ips calligraphus* and *Dendroctonus mexicanus* are found on many species of pine. *Pinus maximinoi* and *P. pseudostrobus* are the hosts of both species of insects. Little research has, however, 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* spp. associated with the two beetle species. In total, six *Ophiostoma* spp. were found to be associated with them and of which *O. nigrocarpum* is recorded from Mexico for the first time. The collection also included a previously undescribed species isolated from both beetles, and for which we provide the name *O. pulvinisporum*.

Keywords: *Ips*, *Dendroctonus*, rRNA.

INTRODUCTION

Pinus spp. 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* spp. occurs in Central and North American countries such as Mexico (Price, Liston & Struss, 1998). Many bark beetle species (Coleoptera: Scolytidae) infest pines. In Mexico, two of the bark beetle species, *Ips calligraphus* (Germar) and *Dendroctonus mexicanus* (Hopkins), occur on indigenous *Pinus* spp. (Wood & Bright, 1992). *Dendroctonus 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), while *I. calligraphus* is known to infest six species of pine, in some cases as a primary insect but in others as secondary agent, with *P. maximinoi* as one of its most common hosts in tropical environments.

A large number of bark beetle species are considered as pests, also because they act as vectors of fungi, particularly ophiostomatoid fungi (Münch, 1907; Whitney, 1982; Beaver, 1989; Paine, Raffa & Harrington, 1997). Some ophiostomatoid species are important plant pathogens (Harrington, 1988; Brasier & Mehrotra, 1995), and many are the causal agents of sapstain (Lagerberg, Lundberg, & Melin, 1927; Seifert, 1993). To the best of our knowledge, at least 14 ophiostomatoid species have been reported from Mexico in five research papers (Table 1). Recently, we had the opportunity to examine bark beetles and their galleries from Mexico, and to isolate ophiostomatoid fungi occurring on the beetles and in the galleries. The aim of this study was to identify the *Ophiostoma* spp. associated with two beetle species. Light microscopy and sequence 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. In Chiapas, Mexico, 35 galleries of *D. mexicanus* infesting dying *P. pseudostrobus*, and 20 of *I. 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 a fine sterile needle and transferred to a medium selective for *Ophiostoma* spp. (20 g Biolab malt extract, 20 g Biolab agar and 1000 ml distilled water, amended with 0.05 % cycloheximide and 0.04 % streptomycin).

Beetles from the same gallery 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 distilled 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, examined microscopically, and characteristic structures were measured. Isolates with *Hyalorhinocladiella* anamorphs resembling the anamorph of *O. ips* (Rumbold) Nannfeldt, were also grown on 2 % WA (20 g Biolab agar and 1000 ml distilled water), with sterilised pine twigs, to induce production of perithecia. For *O. ips*-like cultures, 50 measurements were made for each structure, and the ranges and averages were computed.

Growth studies

The optimal growth temperature for selected isolates resembling the anamorph of *O. ips* (CMW 9023, CMW 9024, and CMW 9028) was determined by growing the isolates at temperatures ranging from 5 to 35 °C, at 5 °C intervals. Each isolate was inoculated onto the agar surface in six 2 % MEA plates for each temperature, with a 6.0 mm diameter agar disk taken from the actively growing margin of a fresh isolate. Colony diameters were measured after four and eight days, and an average was calculated from six random measurements. Growth rates of three authenticated *O. ips* isolates (CMW 6445, CMW 9013, and CMW 9319) were determined in a similar way.

Mating experiments

Mating experiments were conducted on isolates from Mexico resembling *O. ips* (CMW 9024 and CMW 9028) 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 °C 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 single conidium cultures to confirm the authenticity of the tester strains. The thallism of authentic *O. ips* isolates (CMW 6418 and CMW 6463) were determined in a similar way, except that 30 single ascospore and 30 single conidium cultures were made of each isolate.

DNA sequencing and phylogenetic analysis

Some isolates only reproduced asexually in culture and were difficult to identify based on morphology. Of these, 12 single hyphal tip isolates resembling *Sporothrix* or *Hyalorhinocladiella*, were selected for sequencing (Table 2). Since some isolates resembled the anamorph of *O. ips*, and only one reference sequence for this species was available from GenBank, ten authentic isolates of *O. ips* originating from other parts of the world were also included in the study (Table 3). DNA was extracted using a modified version of the extraction method developed by Raeder and Broda (1985). PCR amplification, DNA Sequencing, and phylogenetic analysis were conducted in a similar way as described in Chapter 3. The trees were rooted using the GenBank sequence of *O. ulmi* (Buisman) Nannf. (AF198232) for the *Hyalorhinocladiella* group, and sequence of *O. stenoceras* (Robak) Nannf. (AF484462) for the *Sporothrix* group. Bootstrap analysis (1000 replicates) was run to determine confidence intervals of the branching points.

RESULTS

Isolation of fungi

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

Morphological studies

Morphological study of isolates showed that five ophiostomatoid species, *Ceratocystiopsis minuta* Upadhyay & Kendrick, an *Ophiostoma galeiformis*-like sp., *O. pluriannulatum* (Hedgc.) H. & P. Sydow, a *Hyalorhinocladiella* sp., and *Sporothrix* spp., were associated with *D. mexicanus*. Two

species, *O. pluriannulatum* and a *Hyalorhinocladiella* sp., were collected from the galleries of *I. calligraphus*.

Growth studies

The *O. ips*-like isolates from Mexico grew optimally at 30 °C, while authentic strains of *O. ips* grew best at 25 °C, reaching 58 mm and 50 mm in diameter in four days, respectively. No growth was obtained for any of the isolates at 5 °C, and minimal growth occurred at 10 and 35 °C.

Mating experiments

None of the 10 single ascospore or 20 single conidium cultures of the *O. ips*-like isolates produced perithecia. However, when crossed with each other, 30 of the 45 crosses produced perithecia. Two most vigorously growing strains were selected for mating between isolates. Twenty of 30 single ascospore cultures (67 %), and 24 of 30 single conidium cultures (80 %) of *O. ips* produced perithecia.

Sequence analysis

The *Sporothrix* group. DNA fragments approximately 540 bp in size were amplified for the isolates with *Sporothrix* anamorphs (Table 2). Manual alignment of these sequences resulted in a total of 545 characters. Of these, 8 were parsimony-informative, 25 parsimony-uninformative, and 512 constant. Heuristic searches using *O. stenoceras* as the outgroup taxon, resulted in one most parsimonious tree (Fig. 1) with a length of 37 (CI = 0.946, RI = 0.895, HI = 0.054), in which two main clades were evident. The first clade, which had a bootstrap support of 93 %, included sequences of two isolates (AF484452 and AF484474) of authenticated *O. nigrocarpum* (Davidson) de Hoog, and four Mexican isolates (CMW9485, CMW9486, CMW9488, and CMW9491). The second clade, which had the bootstrap support of 83 %, included two Mexican isolates

(CMW9487 and CMW9489), which are distinct from *O. abietinum* Marmolejo & Butin, *O. stenoceras*, and the other Mexican isolate (CMW9492).

The *Hyalorhinocladiella* group. DNA fragments of approximately 590 bp in size were amplified for the isolates with *Hyalorhinocladiella*-like anamorphs. Manual alignment of these sequences resulted in a total of 624 characters. Of these, 24 were parsimony informative, 115 parsimony uninformative, and 485 constant. Heuristic searches using *O. ulmi* as the outgroup taxon resulted in four most parsimonious trees (CI = 0.968, RI = 0.936, HI = 0.032). Two main clades (Fig. 2) were evident in all phylogenetic trees. The first clade represented the *O. ips* group, with a bootstrap support of 99 %. The second clade, with a bootstrap support of 100 %, represented an undescribed taxon.

TAXONOMY

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

THE FOLLOWING DESCRIPTION SHOULD BE SEEN AS A DRAFT FOR THE PURPOSES OF THIS THESIS AND SHOULD NOT BE CITED. THE COMPLETE DESCRIPTION WILL BE PUBLISHED ELSEWHERE.

Ophiostoma pulvinisporum X. D. Zhou & M. J. Wingfield sp. nov. Figs. 3A - 3G.

Etym.: derived 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 perithecorum globosae, atratae, (150-) 276 (-400) μm diametro, hyphis aseptatis laete griseis ornatae; colla

atrobrunnea vel nigra, laevia, (400-) 1520 (-3520) μm longa, basin versus (28-) 50 (-80) μm , apicem versus (10-) 17 (-48) μm lata. Hyphae ostiolares desunt; asci non visi. Ascospores hyalinae, aseptatae, vaginatae, a latere fronteque visae pulviniformes, (3-) 4 (-6) x (1-) 2 (-3) μm , ab extremo visae quadrangulares.

Anamorpha *Hyalorhinocladiella* dominans, conidiophoris 70 – 160 (- 230) μm longis, conidiis hyalinis, ellipsoideis vel ovoideis, (3-) 6 (-21) x (1-) 2 (-4) μm . Anamorpha *Leptographium* conidiophoris 60 – 120 (- 170) μm longis, conidiis hyalinis, oblongis vel ellipsoideis, basibus truncatis, (2-) 4 (-6) x (1-) 1.5 (-2.5) μm . Anamorpha *Pesotum* conidiophoris 240 – 260 (- 280) μm longis, conidiis hyalinis, bacillaribus, (3-) 4 (-7) x (1-) 1.3 (-2.5) μm .

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

Anamorphs: *Pesotum* (Fig. 3D), *Leptographium* (Fig. 3E), and *Hyalorhinocladiella* (Fig. 3F, 3G). Perithecia produced superficially on 2 % WA when strains of opposite mating type are crossed. Perithecial bases globose, dark, (150-) 276 (-400) μm in diameter (Fig. 3A), ornamented with aseptate light grey hyphae. Perithecial necks dark brown to black, smooth, (400-) 1520 (-3520) μm long, (28-) 50 (-80) μm wide at base, (10-) 17 (-48) μm wide at the apex (Figs.3A, 3B). Ostiolar hyphae absent. Asci not observed. Ascospores hyaline, aseptate, with sheaths, pillow shaped in side and face view, (3-) 4 (-6) x (1-) 2 (-3) μm (Fig. 3C), quadrangular in end view.

Hyalorhinocladiella anamorph predominant, conidiophores: 70 – 160 (- 230) μm long; conidia hyaline, ellipsoid to ovoid, (3-) 6 (-21) x (1-) 2 (-4) μm . *Leptographium* anamorph: conidiophores 60 – 120 (- 170) μm long, conidia hyaline, oblong to ellipsoid with truncate bases,

(2-) 4 (-6) x (1-) 1.5 (-2.5) μm . *Pesotum* anamorph: conidiophores 240 – 260 (- 280) μm long, conidia hyaline, rod-shaped, (3-) 4 (-7) x (1-) 1.3 (-2.5) μm .

Colonies with optimal growth at 30 °C on 2 % MEA, reaching 58 mm in diameter in four days. Colonies light grey (19''d) to dark mouse grey (13''''k) with age (Rayner, 1970). No growth at 5 °C, and minimal growth at 10 and 35 °C.

Specimens examined: Cultures on 2 % MEA, isolated from *D. mexicanus* infesting *P. pseudostrobus*, Chiapas, Mexico, February, 2001, collected by M. J. Wingfield, CMW 9022 (Holotype: PREM***, dried culture). Paratypes: isolated from *D. mexicanus* infesting *P. pseudostrobus*, Chiapas, Mexico, February, 2001, collected by M. J. Wingfield, CMW 9020 (Paratype: PREM***, dried culture) ; isolated from *I. calligraphus* infesting *P. maximinoi*, Chiapas, Mexico, February, 2001, collected by M. J. Wingfield, CMW 9026 (Paratype: PREM***, dried culture) and CMW 9493 (Paratype: PREM***, dried culture).

DISCUSSION

In this study, six ophiostomatoid species were found associated with *D. mexicanus* and *I. calligraphus* from Mexico. These included *Cop. minuta*, *O. pluriannulatum*, an *O. galeiformis*-like species, *O. nigrocarpum* and two species closely related to, but distinct from *O. nigrocarpum*, as well as the new species similar to *O. ips* that we have named *O. pulvinisporum*. Apart from *O. pulvinisporum*, *O. nigrocarpum* is also recorded for the first time from Mexico.

Ceratocystiopsis minuta was first described by Siemaszko from *Picea abies* infested by *Ips typographus* (Linnaeus) in Poland (Siemaszko, 1939). The fungus is commonly associated with *I. typographus* infesting Norway spruce, *Tomicus* spp. infesting *Pinus* spp. in Europe (Mathiesen-

Käärik, 1953; Solheim, 1986), as well as with various conifer-infesting *Dendroctonus* and *Ips* spp. 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* (Herbst) 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.

Ophiostoma 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 *P. 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 *Ips calligraphus*.

Ophiostoma galeiformis (Bakshi) Mathiesen-Käärik was first described by Bakshi (1951) in 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 since the type material has apparently been lost. The identity of the isolate from Mexico will thus be considered in a future study focussing on isolates of resembling this species from different parts of the world.

Ophiostoma nigrocarpum was first described by Davidson (1966) from *P. ponderosa* infested with *D. brevicomis* (LeConte) in the USA. The fungus has been reported on both conifers and hardwoods and has been collected in North America, New Zealand and Australia (Schirp *et al.*, 1999). Ribosomal DNA sequence data have, however, shown that what is presently regarded as the *O. nigrocarpum*-complex consists of a number of distinct taxa (De Beer *et al.*, 2003). A fungus that has been referred to as *O. abietinum* Marmolejo & Butin, originally described from *Abies vejari* in Mexico (Marmolejo & Butin, 1990), is also included in this complex (De Beer *et al.*, 2003). Comparison of DNA sequence data in the present study showed that four Mexican isolates (CMW9491, CMW9486, CMW9488, CMW9485) from *D. mexicanus* infesting *P. pseudostrobus*, grouped together with two *O. nigrocarpum* isolates (CBS408.77 and ATCC22391), isolated from *P. ponderosa* and a *Dendroctonus* sp. respectively in the USA. This group had strong bootstrap support of 93 % and we believe it represents *O. nigrocarpum*. Two other Mexican isolates from *P. pseudostrobus* (CMW9489 and CMW9787) formed a clade distinct from the remaining isolates, with bootstrap support of 83 %. The single isolate (CMW9492) from *P. maximinoi*, also grouped separately from *O. nigrocarpum*, as did *O. abietinum*. Our results, therefore, suggest that *O. abietinum*, the isolate from *P. maximinoi*, and the two *P. pseudostrobus* isolates grouping separately, represent taxa distinct from *O. nigrocarpum*. Further studies, including additional isolates, will be needed to clarify the identity of these fungi.

Ophiostoma pulvinisporum and *O. ips* are morphologically similar. Both *O. pulvinisporum* and *O. ips* are characterised by pillow-shaped ascospores with distinct sheaths. The anamorphs of both 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. ips* have in the past been referred to the genera *Hyalorhinocladiella*, *Graphium* (now *Pesotum*), and *Leptographium*, since no single genus can accommodate the variety of structures produced by this species (Rumbold, 1931, 1941; Hunt,

1956; Davidson, 1978; Upadyhay, 1981; Wingfield, Seifert & Webber, 1993). In our opinion, the *Hyalorhinocladiella* form of *O. pulvinisporum* is predominant and if an anamorph genus is required, we would preferentially refer to it as *Hyalorhinocladiella*.

Ophiostoma 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 °C and *O. ips* at 25 °C. Our results also showed that *O. pulvinisporum* is clearly heterothallic. In contrast, *O. ips* is considered homothallic. ITS rDNA sequence data comparisons in this study, furthermore, supported the separation of *O. pulvinisporum* and *O. ips*.

Ophiostoma 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* sp. 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*, but this can only be confirmed if cultures from the original studies are obtained.

The presence of a large number of 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 also to be expected since 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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Table 1. Ophiostomatoid fungi reported from Mexico.

Fungal species	Host	Insect	^a Ref.
<i>Ophiostoma abietinum</i> Marmolejo & Butin	<i>Abies vejari</i>	<i>Pseudohylesinus</i> sp.	1, 2, 3
<i>O. conicolum</i> Marmolejo & Butin	<i>Pinus cembroides</i>	<i>Conophthorus cembroides</i>	1, 2, 3
<i>O. hyalothecium</i> Davidson	<i>P. pseudostrabus</i>		1
<i>O. ips</i> (Rumbold) Nannfeldt	<i>P. teocote</i> ;	<i>Dendroctonus mexicanus</i> ; <i>Ips</i>	1
	<i>P. pseudostrabus</i>	sp.	3
<i>O. minus</i> (Hedgcock) H. & P. Sydow	<i>P. arizonica</i>		3
<i>O. piceae</i> (Münch) H. & P. Sydow	<i>Quercus affinis</i>		3
<i>O. piliferum</i> (Fries) H. & P. Sydow	<i>P. hartwegii</i>		3
<i>O. pluriannulatum</i> (Hedgcock) H. & P. Sydow	<i>Q. affinis</i> ; <i>P. pseudostrabus</i>		3
^b <i>Ceratocystis tubicollis</i> Olchow. & Reid	<i>P. teocote</i>	<i>D. valens</i>	1
<i>C. adiposa</i> (Butler) Moreau	Soil		4
<i>Cop. vulliferu</i> Marmolejo & Butin	<i>P. teocote</i> ; <i>P. hartwegii</i>	<i>D. valens</i>	1, 2, 3
<i>Cop. fasciata</i> (Wright & Cain) Upadhyay	<i>P. pseudostrabus</i>	<i>D. mexicanus</i>	1, 3
<i>Cop. minuta</i> (Siem.) Upadhyay & Kendrick	<i>P. pseudostrabus</i>	<i>D. mexicanus</i>	1, 3
<i>Sporothrix schenckii</i> Hekt. & Perkins	Human		5

^a References: 1. Marmolejo-Moncivais, 1989; 2. Marmolejo & Butin, 1990; 3. Marmolejo & García-Ocañas, 1993; 4. Reyes & Castillo, 1981; 5. Travassos & Lloyd, 1980.

^b Should be transferred to *Ophiostoma* according to Wingfield, Seifert & Webber, 1993.

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

Species	Host	Insect	^a Isolation Number
<i>Ceratocystiopsis minuta</i>	<i>Pinus pseudostrabus</i>	<i>Dendroctonus mexicanus</i>	CMW10771
<i>Ophiostoma galeiformis</i> -like	<i>P. pseudostrabus</i>	<i>D. mexicanus</i>	CMW9490
<i>O. nigrocarpum</i>	<i>P. pseudostrabus</i>	<i>D. mexicanus</i>	^b CMW9485; CMW9486; CMW9488; CMW9491
<i>O. nigrocarpum</i> -like 1	<i>P. maximinoi</i>	<i>Ips calligraphus</i>	^b CMW9492
<i>O. nigrocarpum</i> -like 2	<i>P. pseudostrabus</i>	<i>D. mexicanus</i>	^b CMW9487; CMW9489
<i>O. pluriannulatum</i>	<i>P. pseudostrabus</i>	<i>D. mexicanus</i>	CMW10772
	<i>P. maximinoi</i>	<i>I. calligraphus</i>	CMW10773
<i>O. pulvinisporum</i>	<i>P. pseudostrabus</i>	<i>D. mexicanus</i>	^c CMW9020; CMW9022; CMW9023
	<i>P. maximinoi</i>	<i>I. calligraphus</i>	CMW9024; CMW9026; CMW9028; CMW9493

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

^b Isolate with *Sporothrix* anamorphs.

^c Isolates with *Hyalorhinocladiella* anamorphs.

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

Species	^a Isolate	Other no.	GenBank	Collector/supplier	Origin	Host/insect
<i>O. abietinum</i>		CBS125.89	AF484453	JG Marmolejo	Mexico	<i>Abies vejari</i>
<i>O. ips</i>		C327	AF198244	TC Harrington	USA	-
	CMW312			P Bedker	USA	<i>Pinus resinosa</i>
	CMW1173			Mendel	Israel	<i>Crypturgus mediteranous</i>
	CMW5089				Chile	<i>P. radiata, H. ligniperda</i>
	CMW6402				Chile	<i>P. radiata, H. ligniperda</i>
	CMW6418				South Africa	<i>P. elliottii, O. erosus</i>
	CMW6463				South Africa	<i>P. elliottii, H. ligniperda</i>
	CMW7075	CBS137.36		CT Rumbold	USA	<i>Ips integer</i>
	CMW7076	CBS151.54		A Käärrik	Sweden	<i>O. proximus</i>
	CMW7079	CBS438.94		T Kirisits	Austria	<i>I. sexdentatus</i>
	CMW9005				Sweden	<i>P. sylvestris, I. acuminatus</i>
<i>O. nigrocarpum</i>		CBS408.77	AF484452	HS Whitmey	USA	<i>P. ponderosa</i>
		ATCC22391	AF484474	RW Davidson	USA	<i>Dendroctonus</i> sp.
<i>O. stenoceras</i>	CMW3202	CBS237.32	AF484462	H Robak	Norway	Pine pulp
<i>O. ulmi</i>		CBS102.63	AF198232	FW Holmes, HM Heybroek	Netherlands	<i>Ulmus hollandica</i>

^a CMW = Culture Collection Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Fig. 1. Phylogram of the *Sporothrix* group based on analyses of ITS sequences (ITS1 and ITS2 regions, as well as 5.8S rRNA gene). *Ophiostoma stenoceras* was used as outgroup. Base substitution numbers are indicated above the branches and the bootstrap values (1000 bootstrap repeats) below the branches.

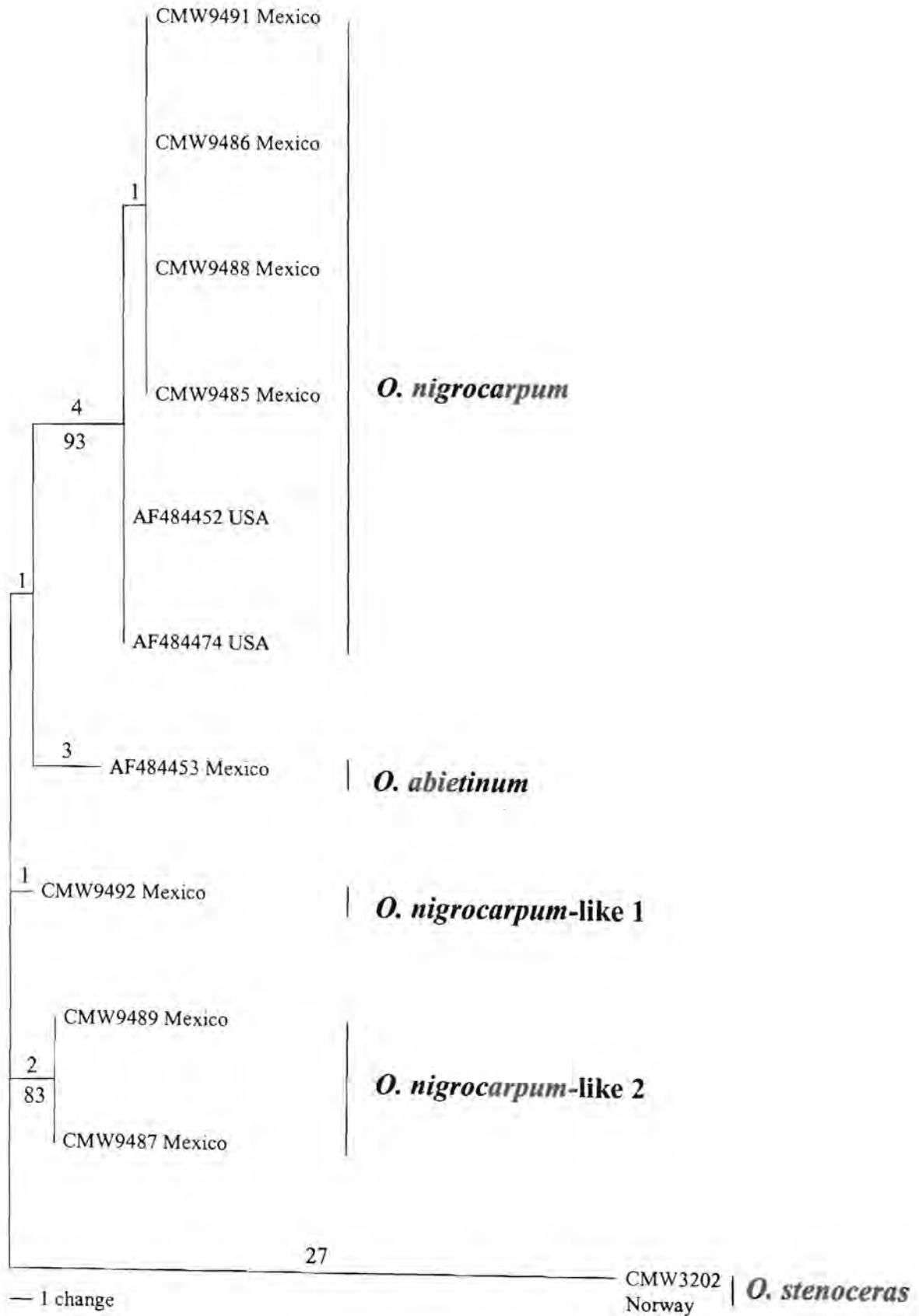
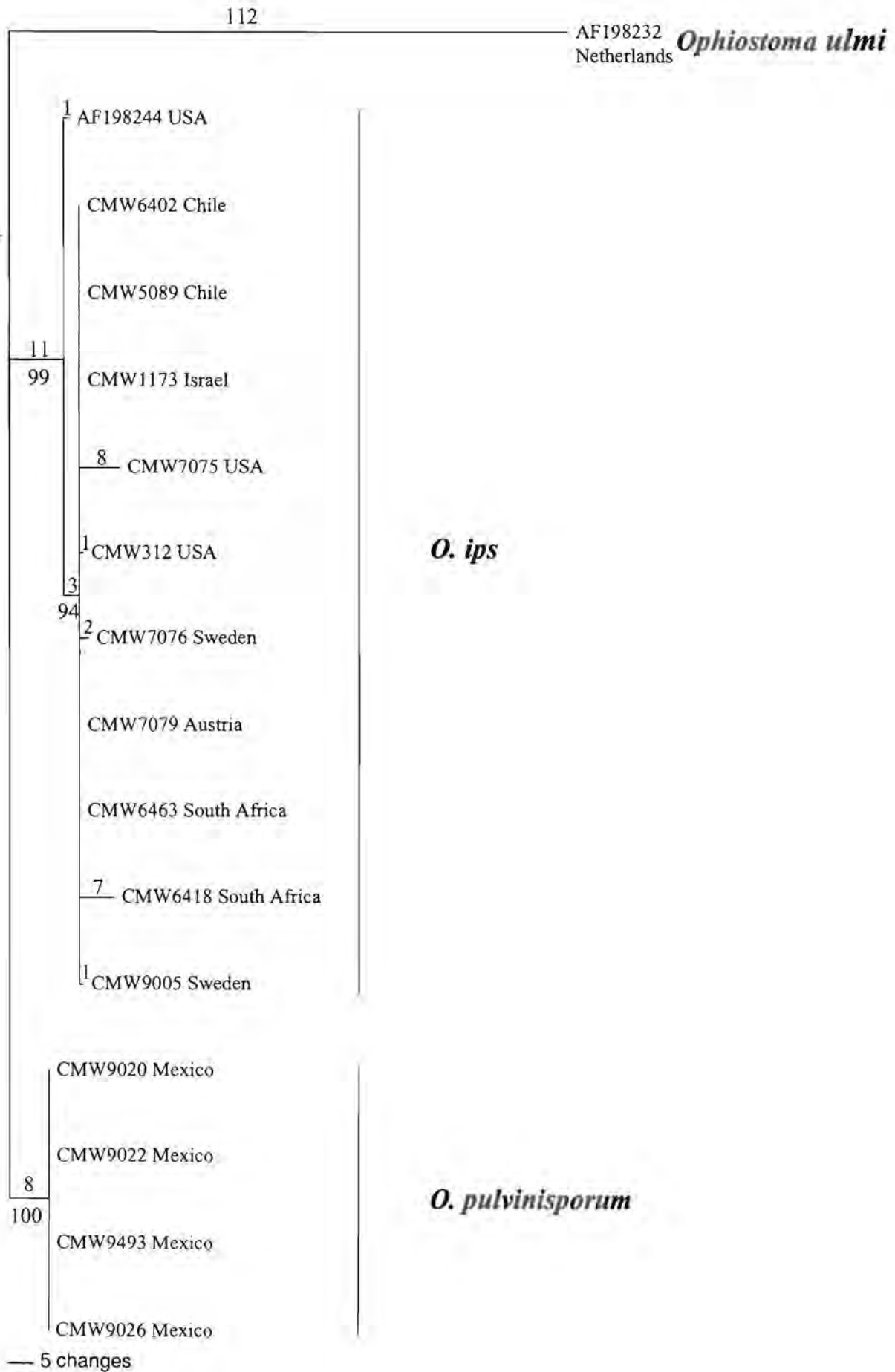
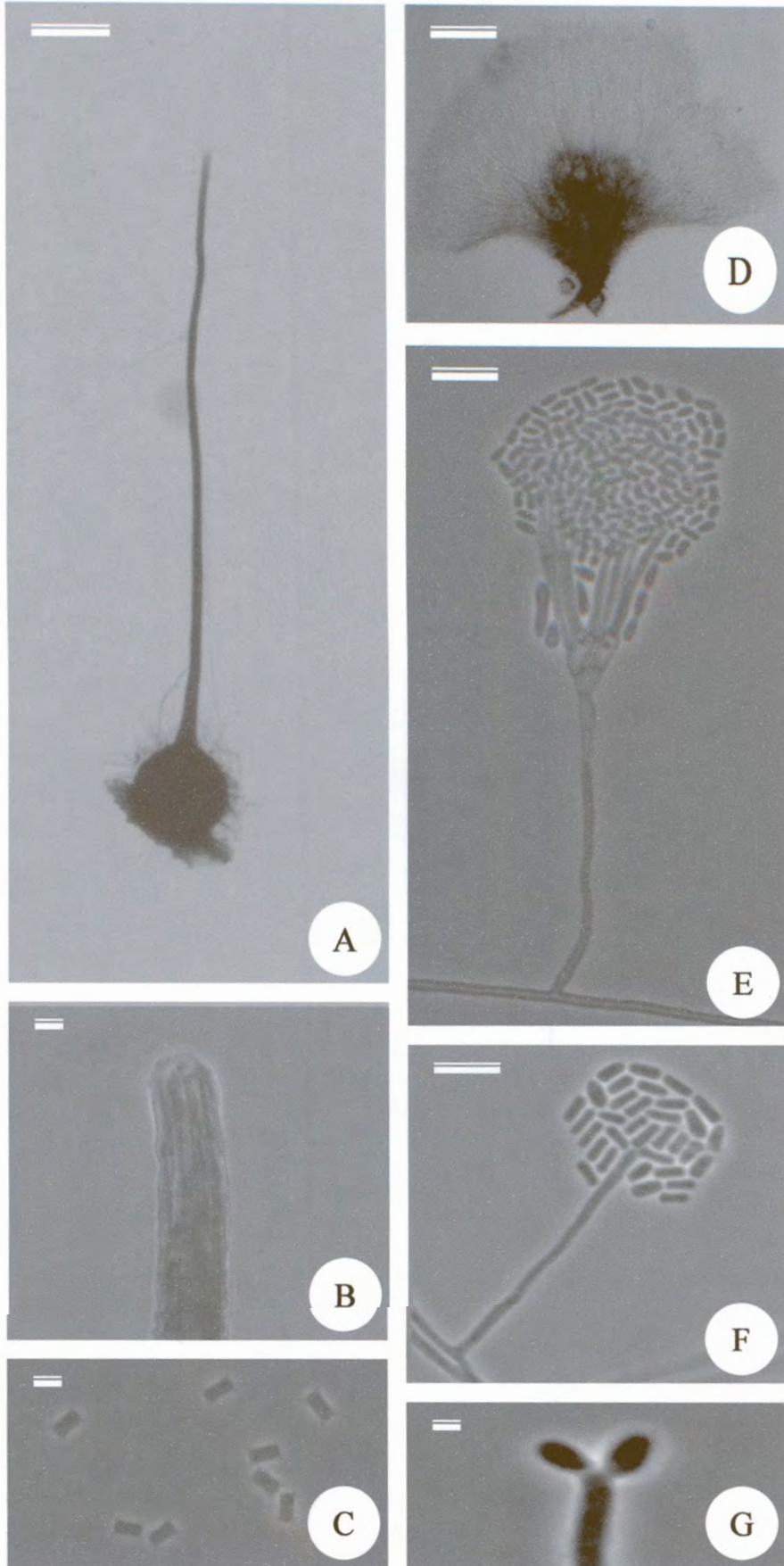


Fig. 2. Phylogram of the *Hyalorhinocladiella* group based on analyses of ITS sequences (ITS1 and ITS2 regions, as well as 5.8S rRNA gene). *Ophiostoma ulmi* was used as an outgroup. Base substitution numbers are indicated above the branches and the bootstrap values (1000 bootstrap repeats) below the branches.



Figs. 3A - 3G. *Ophiostoma pulvinisporum* (CMW 9022) on 2 % MEA. A. Dark perithecia with long neck (Bar = 210 μm). B. Apex of the neck without ostiolar hyphae (Bar = 8 μm). C. Pillow shaped ascospores (Bar = 4 μm). D. *Pesotum* anamorph (Bar = 11 μm). E. *Leptographium* anamorph (Bar = 17 μm). F. *Hyalorhinocladiella* anamorph (Bar = 9 μm). G. Conidia of *Hyalorhinocladiella* anamorph (Bar = 2 μm).





Chapter 5

A new *Leptographium* species associated with *Tomicus piniperda* in South-western China*

Tomicus species (Coleoptera: Scolytidae) are serious pests of pines with a wide distribution in Europe, Asia and America. In Yunnan, South-western China, *T. piniperda* has destroyed more than 0.5 million ha of *Pinus yunnanensis* in the past 15 years. A blue stain fungus belonging to the genus *Leptographium* is associated with both the shoot-feeding and trunk-attacking stages of the beetle's life cycle. The fungus is morphologically similar to the anamorph of *Ophiostoma crassivaginatatum* and to *L. pyrinum*, which are both characterised by short robust conidiophores and hyphae covered by a granular layer. Both these species have been isolated from conifers and are associated with insects. After comparing the fungus from *T. piniperda* with similar *Leptographium* species, using light and scanning electron microscopy, we concluded that it represents a new taxon, which is described here as *L. yunnanense* sp. nov.

Keywords: morphology, taxonomy, *Pinus yunnanensis*, Yunnan.

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INTRODUCTION

Tomicus piniperda L. (Coleoptera: Scolytidae), one of the world's major pine forest pests, is widely distributed throughout Europe, North America and Asia (Bakke, 1968; Haack & Kucera, 1993; Yin, Huang & Li, 1984). In Europe, it is the principal bark beetle that attacks Scots pine (*Pinus sylvestris* L.) and is responsible for severe growth losses (Långström & Hellqvist, 1990). Recently, the beetle has been reported in North America, where it has contributed to a decline in quality of Christmas trees in the Great Lake region (Haack & Lawrence, 1997). In China, it is regarded as one of five major pests of pines. In the past 15 years, it has destroyed more than 0.5 million ha of Yunnan pine (*P. yunnanensis* Franchet) (Ye, 1991), representing 52 % of the total 5 million ha of forests.

Bark beetles that infest conifers are commonly known to carry *Leptographium* spp. (Kendrick, 1962; Harrington, 1988; Wingfield & Gibbs, 1991; Wingfield, 1993;). In some cases this association can lead to considerable losses to forestry (Harrington, 1993). The insects appear to be vectors of the fungi, while the fungi might serve as a source of food for the insect and contribute to the death of trees through mycelial penetration and toxin release (Paine, Raffa & Harrington, 1997). The nature of this association is, however, still actively being debated (Wingfield, Harrington & Solheim, 1995).

Leptographium species can generally be recognised by their long mononematous conidiophores with dark stipes and complex conidiogenous apparatuses, consisting of a series of branches. These branches terminate in conidiogenous cells that produce numerous conidia through annellidic conidium development (Kendrick, 1962). In most cases the conidiogenous cells are characterised by delayed secession of the conidia, which gives them the appearance of sympodial conidium development (Van Wyk, Wingfield & Marasas, 1988). The hyaline and

aseptate conidia of *Leptographium* species are accumulated in slimy masses at the apices of the conidiophores, making these fungi ideal for insect dispersal.

Limited research has been done on the taxonomy of fungi associated with bark beetles in Asia (Kaneko & Harrington, 1990; Yamaoka et al., 1997, 1998; Masuya, Kaneko & Yamaoka, 1998; Masuya *et al.*, 1999) and no previous study has been conducted in China. During the course of a study to isolate and identify the blue stain fungi associated with *T. piniperda* attacking *P. yunnanensis* in South-western China, a *Leptographium* species was isolated in association with *T. piniperda*. The aim of this study was to identify and name this fungus.

MATERIALS AND METHODS

Sampling and fungal isolation

In excess of 1200 adult *T. piniperda* beetles were collected during the beetle's shoot-feeding and trunk-attacking stages in four different localities of Yunnan, South-western China, approximately 250 kilometers apart. Collections were made during one season of the beetle's life cycle. Each infested shoot, including a beetle within it, and each gallery including a pair of beetles within the gallery, were placed in a separate clean plastic bag. All beetle samples were inoculated into 1 m long (15-20 cm diam.) freshly cut and uninfected Yunnan pine logs waxed at both ends using the technique described by Furniss, Solheim and Christiansen (1990), and maintained at room temperature. After 4 weeks, fungi were isolated from the lesions that developed in the phloem and transferred to potato dextrose agar (PDA, 150 g potatoes, 15 g dextrose in 1000 ml distilled water) amended with 0.04% streptomycin. Purified colonies were transferred to 2 % malt extract agar (MEA, 20 g Biolab malt extract, 20 g Biolab agar and 1000 ml distilled water) plates and

incubated at 25 °C until the onset of sporulation. Fungal structures for microscopic examination were mounted on glass slides in lactophenol. Fifty measurements of each relevant morphological structure were made. Colours were determined with the aid of colour charts (Rayner, 1970). All cultures used in this study are maintained in the Culture Collection (CMW) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, Republic of South Africa and representative material including dried and living cultures has been deposited in the National Collection of Fungi (PREM, PPRI), South Africa.

Scanning electron microscopy

For scanning electron microscopy, small blocks of agar cut from sporulating colonies were fixed in 3% glutaraldehyde and 0.5 % osmium tetroxide in a 0.1 M phosphate buffer, dehydrated in a graded acetone series and critical-point dried. Specimens were mounted and coated with gold palladium alloy and examined using a JEOL JSM 840 scanning electron microscope.

Optimal temperature for growth

The optimal growth temperature for the isolates (CMW 5152, CMW 5153, CMW 5304 and CMW 5305) was determined by inoculating eight MEA plates for each temperature with a 6.0 mm diam. agar disk taken from the actively growing margin of a fresh isolate. The plates were incubated at temperatures ranging from 5 to 35°C at 5 °C intervals. Colony diameters were measured on the fourth and the eighth day after commencing the experiment, and an average was calculated from eight random readings.

Cycloheximide sensitivity

Cycloheximide tolerance of isolates (CMW 5152, CMW 5153, CMW 5304 and CMW 5305) was determined by growing them on 2 % MEA amended with 0.5 g/l cycloheximide. Dishes

were incubated in the dark at 25 °C for seven days, and two colony diameters were measured at right angles. Five replicate plates were used and the growth rate (mm/day) was determined based on the average of ten diameter readings.

RESULTS

A *Leptographium* species was isolated from 12.3 % of adult *T. piniperda* that had been collected from both shoot-feeding and trunk-attacking stages. Isolates of the *Leptographium* species are characterised by small robust conidiophores and an optimal growth temperature of 25 °C. This species also proved to be tolerant to cycloheximide, with no reduction in growth when grown on 0.5 g/l of the antibiotic. Isolates of this species produced a considerable number of conidia. In older cultures, the spore masses become hardened, making the observation of the sporulating structures difficult. The hyphae of the *Leptographium* species were characterised by granular surfaces. Comparison with known species of *Leptographium* led us to conclude that this is a previously undescribed *Leptographium* species, and it is described herein.

Leptographium yunnanense X. D. Zhou, K. Jacobs, M. J. Wingfield & M. Morelet, sp. nov.

Figs. 1-7.

Coloniae in 2 % MEA ad 25 °C optime crescentes et post 7 dies 13 mm diam attingentes, margine integrae. Mycelia immersa vel emersa, hyphis aeriis sparsis emittentia, atro-olivacea vel hyalina, ad exterius granulares. Conidiophora singula, erecta, macronematosa, mononematosa, 74.0 – 233.0 µm longa; structura rhizoideiformis absens. Apparatus conidiogeni praeter massam conidii (40.0 -) 83.0 - 88.0 (- 127.0) µm longi, ex ramis cylindricis 2 vel 4-seriatis compositi; rami primarii 2 vel 3, pallide olivacei vel hyalini, cylindrici, laeves, 0 - 1-septati, (9.0 -) 12.0 -

15.0 (- 20.0) x 3.0 - 7.0 μm ; rami secundarii pallide olivacei vel hyalini, aseptati, (9.0 -) 13.0 x 15.0 (- 20.0) x 3.0 - 6.0 μm ; rami tertiarii aseptati, 7.0 - 19.0 (- 24.0) x 2.0 - 5.0 μm ; rami quartii (11.0 -) 14.0 - 17.0 (- 20.0) μm . Cellulae conidiogenae discretas, 2 vel 3 per ramum, cylindricas, apicem versus leviter attenuatas, (18.0 -) 23.0 - 26.0 (- 32.0) μm longas, 2.0 - 4.0 (- 6.0) μm laticas. Conidia oblonga vel obovoidea, lasi truncata, ad apicem apparatus conidiogeni in massa guttulata mucilaginoso accumulata, (4.0 -) 7.0 - 8.0 (- 11.0) x 2.0 - 6.0 μm .

Colonies with optimal growth at 25 °C on 2 % MEA, reaching 13 mm in diameter in 7 days; no growth below 10 °C or above 30 °C; able to withstand high concentrations of cycloheximide, with no reduction in growth on 0.5 g/l cycloheximide after 7 days at 25 °C in the dark; on MEA dark olivaceous (19" f), with smooth margins; mycelia submerged or on top of agar with sparse aerial hyphae, dark olivaceous to hyaline, granular outer surface, not constricted at the septa, (2.0 -) 3.0 - 7.0 (- 9.0) μm in diameter. Conidiophores occurring singly, arising directly from the mycelium, erect, macronematous, mononematous, 74.0 - 227.0 (- 233.0) μm in length; rhizoid-like structures absent; stipe light olivaceous, cylindrical, simple, with 0 - 4 septa, occasionally constricted, 11.0 - 66.0 (- 112.0) μm long (from first basal septum to below primary branches), 4.0 - 9.0 μm wide below primary branches; apical cells not swollen, (3.0 -) 5.0 - 6.0 (- 11.0) μm wide at base; basal cells not swollen (Figs. 1, 7a, b). Conidiogenous apparatus (40.0 -) 83.0 - 88.0 (- 127.0) long (excluding the conidial mass), with 2 to 4 series of cylindrical branches; primary branches light olivaceous to hyaline, smooth, cylindrical, with 0 - 1 septum, (9.0 -) 12.0 - 15.0 (- 20.0) μm long, 3.0 - 7.0 μm wide; secondary branches light olivaceous to hyaline, aseptate, (9.0 -) 13.0 - 15.0 (- 20.0) μm long, 3.0 - 6.0 μm wide; tertiary branches light olivaceous to hyaline, aseptate, 7.0 - 19.0 (- 24.0) μm long, 2.0 - 5.0 μm wide; quaternary branches (11.0 -) 14.0 - 17.0 (- 20.0) μm long, 2.0 - 5.0 μm wide (Figs. 2, 7b). Conidiogenous cells discrete, 2 - 3 per branch, cylindrical, tapering slightly toward the apex, (18.0 -) 23.0 - 26.0

(- 32.0) μm long, 2.0 - 4.0 (- 6.0) μm wide. Conidium development occurring through replacement wall building with holoblastic ontogeny, percurrent proliferation and delayed secession, giving the false impression of sympodial proliferation (Minter, Kirk & Sutton, 1982; Van Wyk, Wingfield & Marasas, 1988) (Fig. 4). Conidia accumulating in slimy droplets at the apex of conidiogenous apparatus, oblong to obovoid, with a truncate base, (4.0 -) 7.0 - 8.0 (- 11.0) x 2.0 - 6.0 μm (Figs. 5, 6, 7c).

Materials examined: Cultures on 2 % MEA, isolated from *T. piniperda* infesting *P. yunnanensis*, Yunnan, South-western China, collected by Xu Dong Zhou, Hui Ye and HuaSun Ding, March 1997, CMW 5304 (= PPRI 6907, living culture) (holotype: PREM 56579, dried culture); December 1996, CMW 5305 (= PPRI 6908, living culture) (paratype: PREM 56580, dried culture); March 1997, CMW 5153 (= PPRI 6923, living culture) (paratype: PREM 56578, dried culture); August, 1995, CMW5152 (=PPRI 6909, living culture), no PREM number for this isolate presently.

DISCUSSION

Leptographium yunnanense can easily be recognised by its small conidiophores, which are abundantly produced on the surface of MEA. As with other species in *Leptographium*, *L. yunnanense* can tolerate high concentrations of cycloheximide (Harrington, 1988). *Leptographium yunnanense* is typical of the other members of this genus: i.e., numerous conidia produced through annellidic conidium development and accumulated in slimy masses on the apices of the conidiophores. In older cultures, spore masses flow from the conidiophores,

become sticky and cover the entire structure. This makes the study of the conidiophore structure in older cultures extremely difficult.

Leptographium yunnanense is morphologically similar to the *Leptographium* anamorph of *O. crassivaginatatum* (H. D. Griffin) T. C. Harrington and *L. pyrinum* R. W. Davidson. These species are all characterised by short robust conidiophores and hyphae that appear to have a granular surface. Furthermore, these species have all been isolated from conifers and are associated with insects (Griffin, 1968; Davidson, 1978).

Leptographium yunnanense, lacking the teleomorph, can be distinguished from the anamorph of *O. crassivaginatatum* based on its slightly longer conidiophores (Table 1). *Leptographium yunnanense* and the anamorph of *O. crassivaginatatum* have conidia of similar length, while those of *L. yunnanense* are almost twice as broad as those of *O. crassivaginatatum* anamorph. This makes the conidia of *L. yunnanense* distinctly obovoid compared with the oblong conidia of *O. crassivaginatatum* anamorph (Griffin, 1968).

Leptographium yunnanense can be distinguished from *L. pyrinum* based on the considerably longer conidiophores in the latter species (Table 1). *Leptographium yunnanense* and *L. pyrinum* have conidia of similar dimensions, but can be distinguished based on the pear-shaped conidia of *L. pyrinum*, compared with the obovoid conidia of *L. yunnanense*. *Leptographium pyrinum* is also characterised by conidiophores with rhizoids (Davidson, 1978), while these structures are absent in *L. yunnanense*. In addition, *L. yunnanense* is associated with *T. piniperda* in China, while *L. pyrinum* is associated with *Dendroctonus* species in the western USA (Davidson, 1978).

This is the first report on fungi associated with *T. piniperda* in China. The beetle has been considered to be a secondary pest, which usually colonizes weakened, stressed and recently killed trees (Långström & Hellqvist, 1993). However, in China, it can also attack healthy trees (Ye, 1991). *Leptographium yunnanense* occurs in China, not in Europe, where the beetles infest and

feed on shoots as well as colonise trunks of living trees. The insect, therefore, appears to be a much more serious pest in China than in Europe.

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Table 1. Comparison of morphological characteristics of *Leptographium yunnanense*, *Ophiostoma crassivaginatatum* anamorph and *L. pyrinum*.

	<i>L. yunnanense</i>	<i>O. crassivaginatatum</i> anam.	<i>L. pyrinum</i>
Substrate	<i>Pinus yunnanensis</i> <i>P. densata</i> <i>P. kesiya</i>	^a <i>Picea mariana</i> , <i>P. glauca</i> , <i>Pinus resinosa</i> , <i>P. strobus</i> , <i>P. sylvestris</i> , <i>Populus</i> <i>grandidentata</i> , <i>P. tremuloides</i> , <i>Fraxinus nigra</i>	^c <i>Pinus ponderosa</i>
Insect association	<i>Tomicus piniperda</i>	^b <i>Trypodendron retusus</i>	^d <i>Dendroctonus adjunctus</i>
Conidiophore length	74.0 - 227.0 (- 233.0) μm	25.0 - 106.0 (- 118.0) μm	(117.5 -) 215.0 - 236.5 (- 392.5) μm
Primary branches	2 - 3	2 - 3	2 - 4
Rhizoids	absent	absent	present
Conidium shape	obovoid	oblong to obovoid	oblong, almost pear-shaped
Conidium length	(4.0 -) 7.0 - 8.0 (- 11.0) μm	(4.0 -) 4.5 - 5.5 (- 10.0) μm	5.0 - 12.0 μm
Conidium width	2.0 - 6.0 μm	1.0 - 2.5 μm	4.0 - 6.0 μm
Granular hyphae	present	present	present
Teleomorph	absent	Present	absent

^aGriffin (1968), Olchowecki and Reid (1974);

^bHarrington (1988);

^cDavidson (1978);

^dDavidson (1978), Harrington (1988), Perry (1991), Six and Paine (1996).

Figs. 1 - 6. *Leptographium yunnanense* (CMW 5304) on MEA. Fig. 1. Conidiophores with dark olivaceous stipes and complex conidiogenous apparatuses (Bar = 10 μm), Fig. 2. Complex conidiogenous apparatus (Bar = 20 μm). Figs. 3, 4. Conidiogenous cells showing false sympodial, and annellidic conidiogenesis indicated by arrows (Bar = 10 μm in Fig. 3, 1 μm in Fig. 4). Figs. 5, 6. Conidia (Bar = 10 μm in Fig. 5, 1 μm in Fig. 6).

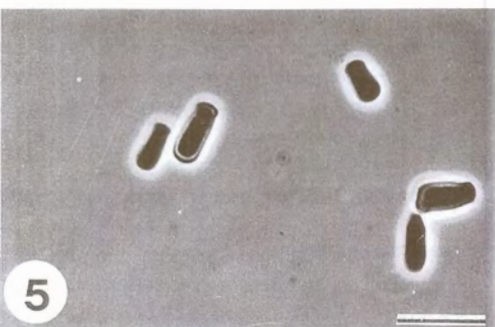
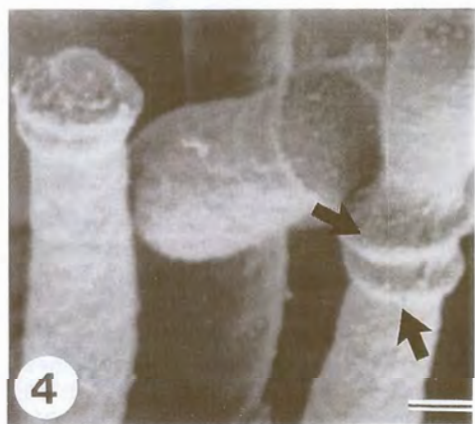
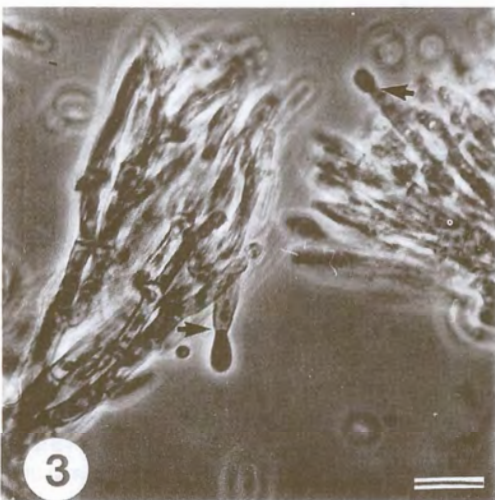
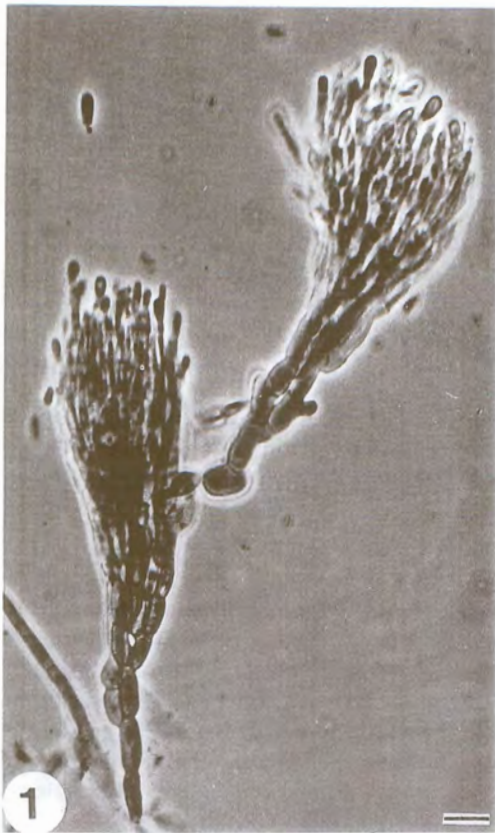
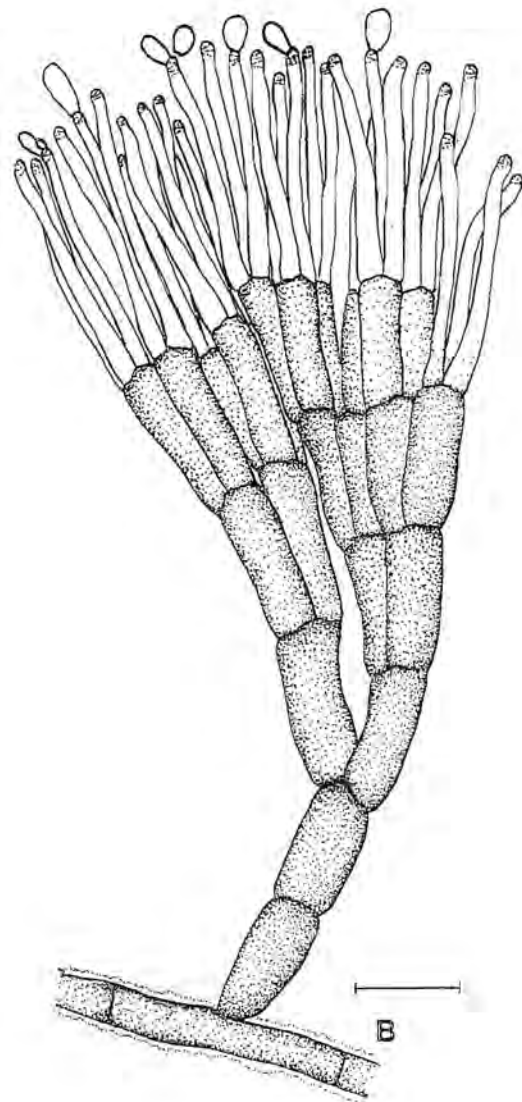
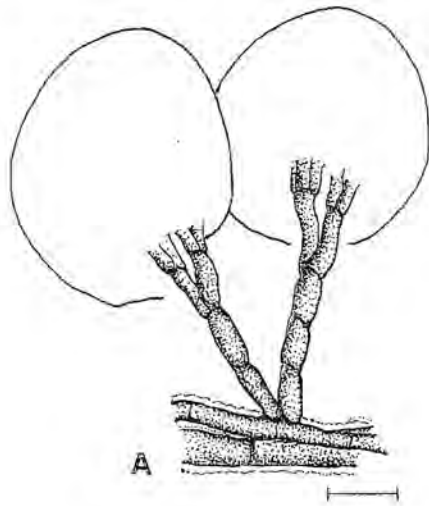
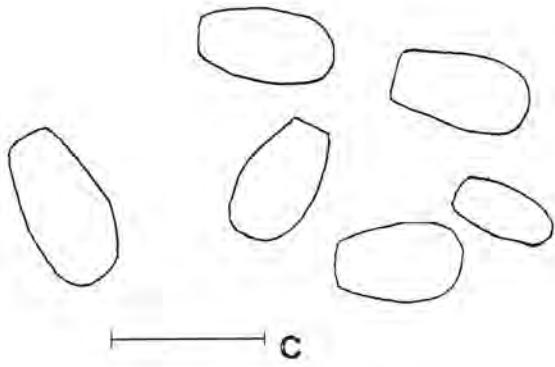


Fig. 7. *Leptographium yunnanense* (CMW 5304) on MEA. A. Habit sketch of the conidiophores (Bar = 50 μm). B. Conidiogenous apparatus (Bar = 10 μm). C. Conidia (Bar = 10 μm).



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