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Ophiostomatales isolated from two European bark beetles, *Hylurgus ligniperda* and *Orthotomicus erosus*, in California

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Ophiostomatales isolated from two European bark beetles, *Hylurgus ligniperda* and *Orthotomicus erosus*, in California

by

Sujin Kim

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vii</td>
</tr>
<tr>
<td>CHAPTER 1. GENERAL INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>Thesis organization</td>
<td>1</td>
</tr>
<tr>
<td>Literature review</td>
<td>1</td>
</tr>
<tr>
<td>Thesis objectives</td>
<td>15</td>
</tr>
<tr>
<td>Literature cited</td>
<td>16</td>
</tr>
<tr>
<td>CHAPTER 2. Ophiostomatales isolated from the root-feeding bark beetle,</td>
<td></td>
</tr>
<tr>
<td><em>Hylurgus ligniperda</em>, in California</td>
<td></td>
</tr>
<tr>
<td>Abstract</td>
<td>30</td>
</tr>
<tr>
<td>Introduction</td>
<td>31</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>33</td>
</tr>
<tr>
<td>Results</td>
<td>37</td>
</tr>
<tr>
<td>Discussion</td>
<td>48</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>55</td>
</tr>
<tr>
<td>Literature cited</td>
<td>55</td>
</tr>
<tr>
<td>CHAPTER 3. <em>Ophiostoma ips</em> and related fungi isolated from a European</td>
<td></td>
</tr>
<tr>
<td><em>Orthotomicus erosus</em>, in California</td>
<td></td>
</tr>
<tr>
<td>Abstract</td>
<td>71</td>
</tr>
</tbody>
</table>
Introduction 72
Materials and Methods 73
Results 77
Discussion 81
Acknowledgements 83
Literature cited 84

CHAPTER 4. GENERAL CONCLUSION 94

ACKNOWLEDGMENTS 96
LIST OF FIGURES

Figure 1. Conidiophores, conidia and hyphae of *Leptographium tereforme*. 70
LIST OF TABLES

Table I. Species of *Ophiostoma*, *Grosmannia* and related anamorph genera associated with *Hylurgus ligniperda* in earlier studies. 66

Table II. Number of male and female adults of *Hylurgus ligniperda* collected live from galleries in the phloem of cut stem-sections of pines (*P. halepensis* and other *Pinus* spp.) at two locations in California. 67

Table III. Number of successful isolations and frequency (percent, in parentheses) and representative ITS and LSU rDNA sequences of species in the Ophiostomatales from 73 adults of *Hylurgus ligniperda* collected at La Cañada Flintridge and 45 adults collected at San Dimas, California. 68

Table IV. Number and frequency of isolation of *Ophiostoma* and related species from adult *Hylurgus ligniperda* collected from New Zealand and USA (California, this study). 69

Table V. Species of *Ophiostoma* and related anamorph genera associated with *Orthotomicus erosus* in earlier studies. 90

Table VI. Location, hosts, and dates collected for male and female *Orthotomicus erosus* adults excavated from infested trees in California. 91

Table VII. Number of successful isolations and frequency (percent, in parentheses) of species in the Ophiostomatales isolated from adults of *Orthotomicus erosus* at four locations in California. 92

Table VIII. Number and frequency of isolations of *Ophiostoma* and related species from adult *Orthotomicus erosus* collected from California (this study), and Spain and South
Africa (earlier studies).
ABSTRACT

Two European pine-infesting bark beetles, *Hylurgus ligniperda* and *Orthotomicus erosus*, were first detected in July 2003 in Los Angeles, California and in May 2004 in the Central Valley of California, respectively. These bark beetles (Coleoptera: Curculionidae: Scolytinae) are common vectors of fungi in the Ophiostomatales, some of which are tree pathogens or causal agents of blue stain of conifer sapwood. Ophiostomatales were isolated on a cycloheximide-amended medium, which is semi-selective for the growth of *Ophiostoma* and related genera. In total, eight identified species and seven unidentified species of Ophiostomatales were isolated from 118 adults of *H. ligniperda* collected from infested pine logs at two sites in California. In the case of *H. ligniperda*, *Ophiostoma ips* and *Grosmannia galeiforme* were isolated from 31% and 23% of the 118 beetles, respectively. The other species isolated included *O. piceae* (isolated from 9% of the beetles), *O. querci* (8%), and a new species that will be described as *Leptographium tereforme* (6%). *Grosmannia huntii*, *L. serpens*, three *Sporothrix* spp., *O. floccosum*, *O. stenoceras*, two unidentified *Hyalorhinocladiella* spp., and a sterile fungus were each isolated from less than 5% of the beetles. In contrast, one identified species and four unidentified species of Ophiostomatales were isolated from 202 adults of *O. erosus* collected from infested pines at four sites in California. *Ophiostoma ips* was isolated from 85% of the 202 adults of *O. erosus*. Beside *O. ips*, a sterile fungus was isolated with 16% frequency, and two *Sporothrix* spp. and a species of *Hyalorhinocladiella* were isolated with fewer than 3% of the *O. erosus*. Most of the identified species were previously known in the USA and have been found in association
with *H. ligniperda* or *O. erosus* in other countries. However, the new species, tentatively named *L. tereforme*, and *G. galeiforme* were recorded from the USA for the first time, and this is the first report of *L. serpens* from western North America.
CHAPTER 1. GENERAL INTRODUCTION

THESIS ORGANIZATION

Chapter one presents a literature review of the association of *Ophiostoma* and related fungal genera with conifer bark beetles. Chapters two and three report on the Ophiostomatales, including *Ophiostoma, Grosmannia, Leptographium* and related anamorph genera, associated with two European bark beetle species (*Hylurgus ligniperda* and *Orthotomicus erosus*) that have been introduced into California. Chapter two focuses on the associations between *Hylurgus ligniperda* and species of *Ophiostoma, Grosmannia,* and *Leptographium* in California and describes a new species of *Leptographium*. Chapter three focuses on the associations between *Orthotomicus erosus* and species of *Ophiostoma* and related anamorph genera in California. Chapter four summarizes the thesis.

LITERATURE REVIEW

I. Taxonomy of bark beetles (Coleoptera: Curculionidae: Scolytinae)

The large family Curculionidae, containing ‘true’ weevils, is generally considered the most highly specialized beetle group, and Curculionidae holds potential for insights into the evolution of insect diversity (Marvaldi et al. 2002, Wood 1986). Within the Curculionidae, the Scolytinae and Platypodinae are the most important subfamilies, containing many important species of bark beetles and ambrosia beetles. Beetles in these groups have a large impact on timber quality, and many bark beetles cause substantial tree mortality, ranking them as the most damaging of all forest insects (Jordal et al. 2008, Marvaldi et al. 2002).

The classification of Scolytinae and Platypodinae has changed frequently during the previous century until Wood's (1986) taxonomic revision of the genera within the Scolytinae. In Wood’s reports (1982, 1986), the Scolytinae and Platypodinae arose together as a monophyletic group from the Curculionidae during the late Triassic to early Cretaceous. The subfamilies Platypodinae, Hylesininae and Scolytinae were considered to represent three main lines of bark and ambrosia beetle evolution (Wood and Bright 1992). Although Platypodinae was believed to be monophyletic, it was considered more closely related to Scolytinae than to Hylesininae based on feeding habits, ecological niches, gallery systems, and social organization (Wood 1982). Building on Wood’s cladistic framework, Kuschel et al. (2000) and Sequeira et al. (2000) supported placing the Platypodinae within the Scolytinae based on morphological characters and DNA sequence analysis. Farrell et al. (2001) also supported the hypothesis that the Platypodinae are derived from the Scolytinae.

The Scolytinae and Platypodinae are known primarily as bark beetles and ambrosia beetles. Bark and ambrosia beetles are defined by distinctive ecological features, including their larval feeding habits (Kuschel et al. 2000, Wood 1982, 1986). The bark beetles in the Scolytinae are mostly phloephagous. They feed directly on the phloem tissues in the inner bark of trees, and both adults and larvae build their own galleries under the bark. On the other hand, the ambrosia beetles that make up most of the Platypodinae and part of the Scolytinae are xylomycetophagous or mycetophagous. Ambrosia beetles construct galleries
in the sapwood, lay their eggs along the galleries, and deposit symbiotic fungi at the same time. Adults of many of the ambrosia beetles have specialized sacs called mycangia, in which their symbiotic fungi grow in a yeast phase. Larvae and adult beetles feed on the symbiotic fungi that grow in the xylem and produce spores in the galleries (Beaver 1989, Six 2003).

Bark beetles are believed to have their origins in the late Cretaceous (67–93 million years before present), feeding on the coniferous genus Araucaria (Farrell et al. 2001, Sequeira and Farrell 2001). The resin canal system of many conifers is believed to be a major defense system against attack from pathogens and other organisms, including bark beetles (Farrell et al. 2001, Paine et al. 1997). The association of the Scolytinae with resin-bearing conifers is one of the most important characteristics of bark beetles. Most bark beetles breed in dead trees, but there are many important tree-killing bark beetles, most of which use an effective aggregation pheromone system to overwhelm the tree’s defense system by attacking individual trees en masse (Franceschi et al. 2005, Paine et al. 1997). Many authors have studied the co-evolution of conifer defenses and bark beetle strategies (Franceschi et al. 2005, Farrell 1988, Farrell et al. 2001, Hulcr et al. 2007, Paine et al. 1997, Raffa and Berryman 1987, Sequeira et al. 2000) and concluded that an association between conifers and bark beetles is an ancestral habit in the Scolytinae. Phylogenetic analyses suggest that the ambrosia beetle habit is derived from the bark beetle habit and evolved at least seven times (Farrell et al. 2001). Most ambrosia beetles attack angiosperms, but even for those that attack conifers, resin is less important as a defense because ambrosia beetles generally attack after the trees are dead or severely weakened by stress.
The Scolytidae are easily transported in freshly cut wood, logs, and packaging material because of their breeding habits (Haack 2003). Thus they are the most commonly intercepted insects found in association with wood products and packing materials, and they continue to spread worldwide, assisted by global trade. For instance, in New Zealand, 84% of the insects intercepted on imports are associated with wood; in the U.S. this figure is 92% (Eckehard et al. 2006, Haack 2003).

II. Taxonomy of Ophiostomatales

Conifer bark beetles are associated with ascomycetous fungi belonging to the order Ophiostomatales in the class Sordariomycetes, including the genera *Ophiostoma*, *Ceratocystiopsis*, and *Grosmannia* as well as related anamorphic genera such as *Pesotum*, *Leptographium*, *Hyalorhinocladiella*, and *Sporothrix* (Harrington 2005, Kiristis 2004). *Ophiostoma* has been considered synonymous with *Ceratocystis* (Hunt 1956, Upadhyay 1981) or distinct from *Ceratocystis* (de Hoog 1974, de Hoog and Scheffer 1984, Harrington 1987, Wingfield et al. 1988) based on morphological and other characters. *Ophiostoma* and *Grosmannia* have been considered synonyms, but recently it has been proposed to recognize *Grosmannia* as distinct (Zipfel et al. 2006). *Ceratocystiopsis* is easily separated from *Ophiostoma* based on falcate ascospores, with ascospore sheaths and short perithecial necks. The taxonomy of the anamorphic states of the Ophiostomatales has also been controversial (Hausner et al. 1993a, c, Spatafora and Blackwell 1993).
Ophiostoma H. Sydow & P. Sydow.—Ophiostoma is the largest genus in the order Ophiostomatales. Within the last 30 years, most taxonomists accepted Ophiostoma and Ceratocystis as synonyms and placed all the Ophiostomatales in one genus, Ceratocystis sensu lato (Hunt 1956, Olchowecki and Reid 1974, Upadhyay 1981). However, de Hoog (1974) and de Hoog and Scheffer (1975) divided Ceratocystis sensu lato into Ophiostoma and Ceratocystis sensu stricto based on two distinct patterns of conidium development. Weijman and de Hoog (1975) found a correlation between Chalara anamorphs and lack of cellulose in cell walls, and they distinguished Ophiostoma and Ceratocystis based on cell wall composition and conidium development: Ophiostoma species contain rhamnose and cellulose in their cell walls and have several anamorphs including Leptographium, Sporothrix, Pesotum, and Hyalorhinocladiella, while Ceratocystis species have cell walls that contain chitin but not cellulose or rhamnose, and have Chalara anamorphs. Harrington (1981) found a correlation between sensitivity to cycloheximide, cell wall chemistry, and anamorphs. He found that the species of Ceratocystis sensu stricto were intolerant of cycloheximide, while species of Ophiostoma were resistant to cycloheximide. De Hoog and Scheffer (1984) confirmed the correlation between hyphal wall chemistry, sensitivity to cycloheximide, and Chalara anamorphs, and introduced Ophiostoma as distinct from Ceratocystis. Harrington (1987) transferred Ceratocystis species with Leptographium anamorphs to Ophiostoma.

Analysis of nuclear encoded small subunit ribosomal RNA gene (SSU rDNA) sequences further supported the separation of Ophiostoma and Ceratocystis (Farrell et al. 2001, Hausner et al. 1993a, c, 2003, Spatafora and Blackwell 1993, Zipfel et al. 2006). Ophiostoma species group closely to the Diaporthales, while Ceratocystis species group with
the Microascales (Spatafora and Blackwell 1993). Farrell et al. (2001) conducted a molecular phylogenetic study of 86 species of Scolytidae and Platypodinae to provide a phylogenetic framework for interactions between beetles, fungi and host plants. They estimated that *Ophiostoma* arose at the same time as the Scolytinae (estimated at 80 million years ago), while *Ceratocystis* is younger than *Ophiostoma* (less than 40 million years old).

The insect relationships of these fungal genera also differ. *Ophiostoma* and related anamorphs often form intimate relationships with ambrosia beetles, bark beetles and weevils, especially coniferous bark beetles (Harrington 1987, 1993b, 2005, Jacobs and Wingfield 2001, Kirisits 2004, Paine et al. 1997). *Ceratocystis* species are associated with a wide variety of insects but rarely with bark beetles (Harrington 1988, 2005, 2009, Kirisits 2004). Therefore, it is now widely accepted that *Ophiostoma* and *Ceratocystis* are distinct genera in separate orders of the Ascomycota.

*Ceratocystiopsis* Upadhyay & Kendrick. — *Ceratocystiopsis* was segregated from *Ceratocystis sensu lato* by Upadhyay and Kendrick (1975). *Ceratocystiopsis* was morphologically characterized by its short ascomatal necks, falcate ascospores with sheaths, and *Hyalorhinocladiella* anamorphs, rather than *Leptographium* or *Pesotum* anamorphs (de Hoog and Scheffer 1984, Upadhyay 1981, Upadhyay and Kendrick 1975). Other than these morphological characters, *Ceratocystiopsis* is very similar to *Ophiostoma*, and synonymy with *Ophiostoma* has been proposed based on morphological and molecular data (Wingfield et al. 1988; Wingfield 1993; Hausner et al. 1993a, c). However, Hsiau and Harrington (1997) reported that *Ceratocystiopsis brevicomi* is closely related to *Ophiostoma* but differs in the
morphology of perithecia and ascospores, and they concluded that *Ceratocystiopsis* is distinct. Hausner et al. (2003) and Zipfel et al. (2006) reviewed *Ceratocystiopsis* using analysis of sequences of ribosomal DNA and the β-tubulin gene, and they also concluded that *Ceratocystiopsis* is distinct from *Ophiostoma*.

*Grosmannia* Goidánich. — *Grosmannia* was erected by Goidánich (1936) for species with *Scopularia* (= *Leptographium*) anamorphs. However, *Grosmannia* was not widely recognized, and most teleomorph species with *Leptographium* anamorphs were treated as species of *Ophiostoma* (Harrington 1987, 1988, Hausner et al. 1993a-c, Jacobs and Wingfield 2001, Seifert and Okada 1993). *Ceratocystis* species with *Leptographium* anamorphs were transferred to *Ophiostoma* by Harrington (1987), and this synonymy was supported by later phylogenetic studies by Hausner et al. (2000) and Zhou et al. (2004b). However, Zipfel et al. (2006) provided phylogenetic analyses of combined nuclear LSU rDNA and β-tubulin genes to support separation of *Grosmannia* from *Ophiostoma*.

Leptographium has had a confused history with many synonyms, including Scopularia, Hantzschia, Phialocephala, and Verticicladiella. Leptographium was established in 1927, based on a single species, L. lundbergii (Lagerberg et al. 1927). Some authors argued against the use of Leptographium in place of Scopularia (Goidàniche 1936), but Leptographium was accepted as the valid name after Shaw and Hubert’s review (1952). In contrast, Hantzschia, which was established for the single species H. phycomyces Auersw., was reduced to synonymy with Leptographium by Shaw and Hubert (1952) but then separated by Hughes (1953) based on their different modes of conidium development: phialidic for Hantzschia and annellidic for Leptographium. After Hughes’s work (1953), many Leptographium-like species were separated and described as Verticicladiella, producing conidia sympodially (Kendrick 1962), and Phialocephala, producing conidia from phialides with periclinal thickening and prominent collarettes (Kendrick 1961, 1963). However, the genus Verticicladiella was not universally accepted as distinct from Leptographium. After Wingfield’s detailed study of conidia production by electron microscopy (1985) and Harrington’s review (1988), Verticicladiella has been considered a synonym of Leptographium.

The genus Phialocephala was originally separated from the Leptographium complex based on production of conidia from phialides (Kendrick 1961, Wingfield et al. 1987). Recently, Jacobs and Wingfield (2001) and Jacobs et al. (2003) evaluated placement of the Phialocephala species with phylogenetic analysis of sequence data and concluded that species of Phialocephala are unrelated to Leptographium or Ophiostoma.
The remaining anamorph genera related to *Ophiostoma* are *Pesotum*, *Sporothrix* and *Hyalorhinocladiella*. The genera *Graphium* and *Pesotum* are characterized by well developed, dark synnemata, producing single celled conidia in slimy masses at their apices (Seifert and Okada 1993). *Graphium* was first described in 1837 by Corda, lectotypified by *G. penicillioides*. Until recently, this genus was thought to have affinity with synnematous anamorphs of *Ophiostoma*, although the teleomorph of *G. penicillioides* was unknown (Seifert and Okada 1993, Upadhyay 1981). However, later studies have shown that the synnematous anamorphs of *Ophiostoma* are not phylogenetically related to *Graphium* species. Okada et al. (1998) showed that *G. penicillioides* and relatives form a monophyletic group within the Microascales, while the synnematous anamorphs of *Ophiostoma* group with the Ophiostomatales. Thus, the genus name *Pesotum*, which was established for the anamorphs of *O. piceae* and *O. ulmi* by Crane and Schoknecht (1973), is available for the synnematous anamorphs of *Ophiostoma*. Harrington et al. (2001) recommended that the name *Pesotum* should be restricted to anamorphs related to the *O. piceae* complex within the Ophiostomatales. They accepted the original description of *Pesotum*, which includes the *Sporothrix* –like forms, and stated that the synnemata of *Ophiostoma* species outside the *O. piceae* complex are loose aggregations of *Leptographium* conidiophores without the fused stipe cells that are characteristic of the *O. piceae* complex.

The genera *Sporothrix* Hektoen & Perkins (1900) and *Hyalorhinocladiella* Upadhyay & Kendrick (1975) are common mycelial anamorphs of *Ophiostoma* species (Upadhyay 1981). *Sporothrix* was described as morphologically similar to the type species *S. schenckii* by de Hoog (1974). *Hyalorhinocladiella* was established from the anamorphs of *Ceratocystis*
s.l. by Upadhyay and Kendrick (1975). These two genera share many morphological characters. Thus, until recently, presence of denticles in *Sporothrix* and smooth conidiogenous cells in *Hyalorhinocladiella* was the only way to distinguish between these two genera (Hughes 1953, Upadhyay 1981). However, more recent studies separate *Sporothrix* from *Hyalorhinocladiella* based on DNA sequences, scanning or transmission electron microscopy, and fluorescence microscopy (Benade et al. 1995, 1997, de Beer et al. 2003). Benade et al. (1997) reported that a unique pattern of conidium development exists in *Sporothrix* and *Hyalorhinocladiella*.

III. Pathogenicity of the Ophiostomatales

The Ophiostomatales contain a few important plant and tree pathogens and some of the most important causal agents of sapstain (Harrington 1993a, Paine et al 1997). At least one species related to *Ophiostoma* (*Sporothrix schenckii*) is a significant human pathogen (Summerbell et al. 1993). Among the most aggressive tree pathogens are *O. ulmi* and *O. novo-ulmi* (syn. *C. ulmi* and *C. novo-ulmi*), which cause Dutch elm disease and have killed millions of elm trees in Europe and North America (Brasier 1991). *Raffaelea lauricola*, which is closely related to *Ophiostoma*, causes laurel wilt in redbay and other members of *Lauraceae* in the lower coastal plains of the southeastern USA (Fraedrich et al. 2008, Harrington et al. 2008). Another aggressive pathogen is *Leptographium wageneri*. Three host-specific varieties of *L. wageneri* are responsible for black stain root disease on conifers, which can kill inoculated seedlings and have led to extensive losses in forests in western North America (Cobb 1988, Harrington 1988, Zambino and Harrington 1989). *Leptographium wageneri* var. *wageneri*

As mentioned above, some *Ophiostoma* species can kill host trees, but most of these fungi are not particularly strong tree pathogens (Harrington 1993a, 2005). However, many of the species associated with bark beetles have been shown to be weakly pathogenic to conifers and have been thought to aid the beetles in killing trees. The less pathogenic species, *O. ips*, *O. minus*, *L. serpens*, *L. terebrantis*, *L. pocrum*, and *L. wingfieldii*, cause stains in the wood of trees and fresh logs. These species are loosely associated with root diseases of pine and may help kill trees if mass inoculated with the mass attacks by the beetles (Eckhardt et al. 2004, Harrington 1988, 1993a, Wingfield et al. 1988, Zhou et al. 2002). Pathogenicity tests of *O. ips*, *L. serpens* and *L. lundbergii* to pine found that *O. ips* is more pathogenic than *L. serpens* or *L. lundbergii*, and *L. serpens* and *L. lundbergii* were not pathogenic to living, healthy pines in South Africa (Zhou et al. at 2002).

IV. Associations between bark beetles and Ophiostomatales

Many authors have reviewed and discussed the associations between bark beetles and Ophiostomatales in live host conifers (Beaver 1989, Harrington 1988, 1993a, b, 2005. Kirisits 2004, Paine et al.1997, Six 2003, Whitney 1982). Aside from the mutualistic associations between some Ophiostomatales and mycophagous bark beetles (Harrington 2005), there is some debate if the Ophiostomatales are commensual or mutualistic with conifer bark beetles. Currently two hypotheses explain this relationship. One hypothesis is that the
relationship is mutualism, where the beetle benefits the fungus by transferring it to new hosts and the fungus benefits the beetle by helping the beetles to kill the tree (Francke-Grosmann 1967, Jacobs and Wingfield 2001, Kirists 2004, Paine et al. 1997, Six 2003, Upadhyay 1981, Whitney 1982). A second hypothesis holds that the relationship is a commensalism where the benefit to the fungus is clear (transmission to new host plants), but the beetles do not benefit (Harrington 1993a, b, 2005, Paine et al. 1997). Although the fungal associates of most tree-killing bark beetles show little capability to colonize a living host plant, most bark beetles attack dead or severely weakened trees, and *Ophiostoma* species apparently invade the sapwood only after the host has died (Harrington 2005). In some cases colonization of the plant host may prove detrimental to the development of the beetle brood (Harrington 2005). Thus, currently, most interaction between bark beetles and Ophiostomatales is considered commensalism.

Bark beetles may be important vectors of the Ophiostomatales because genera in this family produce sticky spore drops, either ascospores or conidia, at the tips of their fruiting structures. Sticky spore drops probably both adhere to the body surfaces of the beetles and are eaten and pass through the insects’ digestive tract (Beaver 1989, Harrington 1993b, 2005, Kirisits 2004, Six 2003). Adults of most bark beetles have pits and crevices in their exoskeletons, and some bark beetles have special fungus-carrying structures call mycangia (Francke-Grosmann 1967) that may be colonized by *Ophiostoma* species, although relatively few members of Ophiostomatales have been shown to be carried in this way (Beaver 1989, Francke-Grosmann 1967, Harrington 2005).
Mycangia are an important character in describing the relationship between bark beetles and fungi (Harrington 2005, Paine et al. 1997) because they are diverse within the scolytid species, varying with sex of the beetle and feeding strategy (xylomycetophagous or phloeophagous) (Francke-Grossmann 1967). Xylomycetophagous ambrosia beetles generally possess mycangia, which disseminate ambrosia fungi. Most xylomycetophagous beetles are associated with the genera *Ambrosiella* or *Raffaelea*, which are polyphyletic, and placed within the large genus *Ophiostoma* based on phylogenetic analyses (Farrell et al. 2001, Harrington 2005, Massoumi et al. 2009). In contrast, only a few phloeophagous bark beetles species have mycangia, especially sac mycangia, because highly developed mycangia may not be essential for the transport of fungi by these bark beetles (Harrington 2005, Six 2003). The most conspicuous associations between bark beetles and fungi are between conifer bark beetles and *Ophiostoma* species or their close relatives. *Ophiostoma* species generally tolerate conifer resin (Farrell et al. 2001) and some species are important to mycophagous bark beetles (Harrington 2005). For example, *O. clavigerum* is important food source for young adults of *D. ponderosae* (Harrington 2005).

Members of Ophiostomatales have relatively specific relationships with beetles (Harrington 1993b, 2005, Jacobs and Wingfield 2001, Kirisits 2004, Paine et al. 1997). For example, *O. clavigerum* is commonly associated with *D. jeffreyi* and *D. ponderosae*, *O. nigrocarpum* with *D. frontalis*, and *O. bicolor, O. penicillatum* and *O. piceaperdum* have developed a specific relationship with *Ips typographus* in Europe (Kirisits 2004). *Ophiostoma ips*, *O. minuta*, *O. huntii*, and *O. terebrantis* are good examples of non-specific
Ophiostomatales that have been associated with numerous bark beetle species (Harrington 2005, Kirisits 2004).

The association between Ophiostomatales and conifer bark beetles has been extensively studied, and several reviews have been written on this topic in different countries. Many studies focused on the fungi isolated from the insects or their galleries and identified fungi to the species level (de Beer et al. 2003, Harrington 2005, Harrington et al. 2001, Hausner et al. 2005, Hutchison and Reid 1988, Paine et al. 1997, Six 2003, Solheim 1986, Wingfield et al. 1988, 1999, Zhou et al. 2004a), and several researchers supported the association between Ophiostomatales and bark beetles and their host plants (Davidson 1953, 1955, Mathre 1964, Reay et al. 2006, Romón et al. 2007, Rumbold 1931, 1936, Zhou et al. 2001). For example, in the U.S., Rumbold (1931, 1936) reported blue-stain fungi associated with *Dendroctonus* spp. and determined associations between the beetles and several fungi. Mathre (1964) surveyed *Ceratocystis* spp. and bark beetles such as *Dendroctonus* and *Ips* in California, and he also reported associations between these fungi and beetles, showing that the beetles serve as vectors for the fungi. In the U.S., associations between Ophiostomatales and bark beetles have been recognized for many years (Davidson 1953, 1955, Harrington 1988, Mathre 1964, Rumbold 1931, 1936, Whitney 1982).

Some limited studies concerning *H. ligniperda* and *O. erosus* and their associations with the Ophiostomatales have been conducted in different countries such as Chile, New Zealand, Spain, and South Africa. Zhou et al. (2004a) reported that *Ceratocystiopsis minuta*, *O. galeiforme*, and *O. ips* were isolated from *H. ligniperda* in Chile. Reay et al. (2006) reported that *O. floccosum*, *O. galeiforme*, *O. huntii*, *O. stenoceras*, *O. ips*, and *L. procerum*
were commonly isolated from *H. ligniperda* in New Zealand. In addition, in Spain, Romón et al. (2007) reported that only *L. guttulatum* was isolated from *H. ligniperda*, but *O. ips*, *O. stenoceras*, *O. pluriannulatum*, and *L. guttulatum* were isolated from *O. erosus*. Similar to the results from Spain, in South Africa, *C. minuta*, *O. galeiforme*, *O. ips*, *O. piceae*, *L. lundbergii*, *L. serpens*, and *Pesotum* sp. were isolated from *H. ligniperda*, and *O. ips*, *O. pluriannulatum*, *L. lundbergii*, *L. serpens*, and *Pesotum* sp. were isolated from *O. erosus* (Zhou et al. 2001a). Based on these results, *O. ips* was the most commonly occurring species found on both *H. ligniperda* and *O. erosus*. However, no research has directly examined whether *H. ligniperda* and *O. erosus* serve as vectors of Ophiostomatales in the U.S.

These two European bark beetles, *Hylurgus ligniperda* and *Orthotomicus erosus*, are distributed in the United States mainly in the California area, especially in Southern California counties (*H. ligniperda*) and the Central Valley counties of California (*O. erosus*) (Lee et al. 2007). *Hylurgus ligniperda* and *O. erosus* are newly-arrived species in the U.S., and now they are among the most commonly intercepted species of Scolytinae coming into the U.S. (Haack 2003), although they are generally considered only secondary pests.

*Hylurgus ligniperda* and *O. erosus* and the fungi they carry have the potential to cause economic losses in the U.S. Aside from direct damage to pine trees, the fungi they carry may cause blue stain or sapstain on timber as they have in other countries such as Chile, New Zealand, and South Africa. Also, these beetles have the potential to introduce new fungal pathogens to U.S. forests.

**THESIS OBJECTIVES**
The objectives of this study were to:

(i) identify the species of Ophiostomatales from *Hylurgus ligniperda* in southern California based on morphological characters, mating studies, and rDNA sequences.

(ii) identify the species of Ophiostomatales from *Orthotomicus erosus* in California based on morphological characters, mating studies, and rDNA sequences.

LITERATURE CITED


Hausner G, Iranpour M, Kim JJ, Breuil C, Davis CN, Gibbe EA, Reid J, Loewen PC, Hopkin AA. 2005. Fungi vectored by the introduced bark beetle *Tomicus piniperda* in Ontario, Canada, and comments on the taxonomy of *Leptographium lundbergii*, *Leptographium*


CHAPTER 2. *Ophiostomatales* isolated from the root-feeding bark beetle, *Hylurgus ligniperda*, in California

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**Abstract:** The root- and stump-feeding bark beetle *Hylurgus ligniperda* is native to Europe but was first discovered in Los Angeles, California in 2003. This bark beetle is a common vector of Ophiostomatales, which are potential tree pathogens or causes of blue-stain of conifer sapwood. In this study, Ophiostomatales were isolated on a cycloheximide-amended medium from 118 adult *H. ligniperda* collected from infested pine logs at two sites in California. In total, eight species of Ophiostomatales were identified, and seven species were unidentified. The most frequently isolated species were *Ophiostoma ips* and *Grosmannia galeiforme*, which were isolated from 31% and 23% of the 118 beetles,
respectively. The other species isolated included *O. piceae* (isolated from 9% of the beetles), *O. querci* (8%), and *Leptographium tereforme* sp. nov. (6%). *G. huntii*, *L. serpens*, three *Sporothrix* species, *O. floccosum*, *O. stenoceras*, two unidentified *Hyalorhinocladiella* sp., and a sterile fungus, were each isolated from less then 5% of the beetles. Most of the identified species were already known in the USA and have been found in association with *H. ligniperda* in other countries. However, the new species, *L. tereforme*, and *G. galeiforme* were recorded from the USA for the first time, and this is the first report of *L. serpens* from western North America.

**Key words:** *Grosmannia galeiforme*, *Grosmannia huntii*, *Leptographium tereforme*, *Leptographium serpens*, *Ophiostoma ips*, *Ophiostoma piceae*, *Ophiostoma querci*, *Sporothrix* spp., *Hyalorhinocladiella* spp., red-haired bark beetle.

**INTRODUCTION**

*Hylurgus ligniperda* (Fabricius) (Coleoptera: Curculionidae: Scolytinae), a pine-infesting bark beetle, is native to Europe but has spread globally in solid wood packing material, dunnage, or logs (Haack 2006). *Hylurgus ligniperda* has been introduced accidentally to Chile, New Zealand, and South Africa, and it was recently introduced into the USA, where it was first detected in 2000 in New York, although specimens had been collected from the same general area as early as 1994. *Hylurgus ligniperda* was also detected in July 2003 in Los Angeles, California (Haack 2006).

*Hylurgus ligniperda* is a root-infesting beetle covered with reddish hairs, hence its common name, the red-haired bark beetle. This beetle has a relatively broad host range,
including pine, spruce, and Douglas-fir. It is generally considered a secondary pest because it does not aggressively kill trees (Haack 2006), although it may become lethal when the trees are stressed or injured (Neumann 1987). The beetle may cause minor economic damage by introducing sapstain fungi into wood (Harrington 1988, Zhou et al. 2004a), and it could become an effective vector of a tree pathogen or blue-stain fungus (Harrington 1988, 1993, 2005, Jacobs and Wingfield 2001, Paine et al. 1997, Six 2003).

Most common blue-stain fungi on conifer sapwood have been classified in the genera *Ophiostoma*, *Ceratocystiopsis*, *Grosmannia*, and *Ceratocystis*. These genera represent two phylogenetically unrelated groups, the Ophiostomatales, close to the Diaporthales, and *Ceratocystis*, in the Microascales (Zipfel et al. 2006). Most blue-stain fungi associated with conifer bark beetles belong in the Ophiostomatales, although a few conifer bark beetles transmit *Ceratocystis* species (Harrington 2005, Kirists 2004). Recently, Zipfel et al. (2006) redefined *Ophiostoma* and distinguished *Ophiostoma* species (with *Pesotum*, *Hyalorhinocladiella*, and *Sporothrix* anamorphs) from *Ceratocystiopsis* (with *Hyalorhinocladiella* anamorphs) and *Grosmannia* (with *Leptographium* anamorphs). Mullineux and Hausner’s (2009) study of the secondary structure of the internal transcribed spacer regions of rDNA further supported separation of *Grosmannia* from *Ophiostoma*.

Sexual species in the Ophiostomatales produce sticky ascospores at the tips of perithecia, and anamorphs of Ophiostomatales typically produce wet droplets of conidia. The sticky spores may be acquired by bark beetles in mycangia or, more commonly, on the exoskeleton (Harrington 1993, 2005, Six 2003). Recent work in Chile (Zhou et al. 2004a), New Zealand (Reay et al. 2006), Spain (Romón et al. 2007), and South Africa (Zhou et al.
2001) report isolating several species of Ophiostomatales from *H. ligniperda*, including *Ceratocystiopsis minuta* (Siemaszko) Upadh. & Kendrick, *O. galeiforme* (Bakshi) Math.-Käärik., *O. ips*, *O. piceae*, *O. querci*, *O. stenoceras*, *L. lundbergii* Lagerb. & Melin, *L. serpens*, and *L. procerum* (W.B. Kendr.) Wingfield (Table I). However, the fungi associated with *H. ligniperda* in the USA have not been documented.

This study explores the relationship between *H. ligniperda* and Ophiostomatales in California to examine whether the beetle may have brought potential tree pathogens or new bluestain fungi to North America. Adult beetles were removed from infested bolts, and *Ophiostoma* species were isolated on cycloheximide-amended media, which is semi-selective for the growth of *Ophiostoma* and related genera (Harrington 1981).

**MATERIALS AND METHODS**

*Beetle collection and isolation* — Pairs of mature adults of *H. ligniperda* were collected live from egg galleries in the phloem (inner bark) of cut stem-sections of pine (*Pinus halepensis* and other *Pinus* spp.) in contact with the ground at Decanso Gardens (La Cañada Flintridge, 34.21055°N, 118.20044°W) and Bonelli Park (San Dimas, 34.08856°N, 117.81253°W) in California on 7 March 2006 (Table II). Adults were individually placed in 1.5 ml microcentrifuge tubes and stored on ice. Sex was determined by dissecting the terminal end of the abdomen for the presence or absence of an aedeagus. Each adult was killed by crushing with forceps and shipped from California to Iowa, where the beetles were kept in a refrigerator until processing. In the laboratory, each beetle was aseptically cut into four parts (head, prothorax, mesothorax and abdomen), and each part was placed into cycloheximide–
streptomycin malt agar (CSMA, 10 g malt extract, 15 g agar, 200 mg cycloheximide, and 100 mg streptomycin per 1L dH₂O). Streptomycin and cycloheximide were added after autoclaving. Plates were incubated at room temperature until colonies became visible on the medium. Cultures were transferred via conidial masses or the edge of the mycelium to fresh plates of 1.5% malt extract agar (MEA, 15 g malt extract and 20 g agar per 1L dH₂O). Pure cultures were used for DNA extraction and morphological examination.

**Fungal morphology.**—Representative isolates of each putative species were stored at −80 C in the collection at Iowa State University. The isolates were grown at room temperature on 1.5% MEA and examined with light microscopy with differential interference contrast (DIC) microscopy (by Normarsky, Olympus BH-12 with Kodak DS 120). All major morphological characters were compared with described Ophiostomatales.

To describe the new species, 10 d cultures were incubated at 25 C on MEA. Thirty measurements were made for each character for each isolate studied, and the ranges and average of each isolate were computed. For a temperature growth study, colonized agar plugs (5 mm diameter) were taken from 10 d cultures and placed in the middle of fresh MEA plates, mycelium side down. The plates were inverted and incubated in the dark at 5, 15, 25, 30 or 35 C. After 10 d, colony radius was calculated by taking two radial measurements from each of three replicate cultures and averaging the six measurements.

**Mating experiments.**—For synnemata-forming species and some *Leptographium* species, representative isolates were paired with tester strains of *O. floccosum* (C989, mat-a, and
C988, mat-b, from New Zealand), *O. galeiforme* (DM269–1, mat-a, and DM269–2, mat-b, from Scotland), *O. huntii* (DM1870, mat-a, from Chile), *O. piceae* (C1618, mat-a, and C1620, mat-b, from Chile), or *O. querci* (C1018, mat-a, and C1017, mat-b, from New Zealand).

Pairings were made on pine twig media (PTM, 1.5% MEA with one piece of autoclaved pine twig) (Harrington 1992). Debarked twigs of *Pinus strobus* L. were cut to 3–4 cm length and autoclaved twice for 50 min. One sterilized twig was placed aseptically in each Petri dish, and autoclaved 1.5% MEA was poured over the twig until covered. In most cases, two isolates were placed next to each other on PTM, and the plates were incubated at room temperature until perithecia appeared. In other cases, an isolate was grown on the PTM until fully colonized, and then the mycelium was spermatized with 20 µl of a conidial slurry of the second isolate (Harrington and McNew 1997).

**DNA sequencing and RFLP analysis.**—For DNA extraction, each isolate was grown at room temperature for 1 wk on malt yeast extract agar (MYEA, 15 g malt extract, 2 g yeast and 20 g agar per 1 L dH₂O), mycelium and spores were scraped from the plate, and DNA was extracted from the scrapings using PrepMan™ Ultra (Applied Biosystems), following the manufacturer’s protocol.

Attempts were made to amplify the 26S rDNA gene (nuclear large subunit, LSU) and the internal transcribed spacer (ITS rDNA) regions of representative isolates of each putative species. The LSU region was amplified using the primers LROR (5′–ACCCGCTGAACCTAAGC–3′) and LR5 (5′–TCCTGAGGGAAAACCTTCG–3′) (Vilgalys and Hester 1990). The ITS regions and the 5.8S gene of the ribosomal RNA operon were
amplified using the primers ITS1-F (5′–CTTGGTCATTTAGAGGAAGTAA–3′) (Gardes and Bruns 1993) and ITS-4 (5′–TCCTCCGCTTATTGATATATGC–3′) (White et al. 1990). The template DNA was amplified in a 50 µl single reaction volume, containing 1.25 units Takara Ex Taq Polymerase, 1X PCR reaction buffer, 200 µM dNTPs, 5% (V/V) DMSO, and 0.25 µM of each primer (Takara Mirus Bio., Japan). Cycling conditions were as follows: initial denaturation at 94 C for 2 min, 35 cycles of annealing at 49 C for LSU and at 54 C for ITS for 35 s, and primer extension at 72 C for 2 min, followed by one final cycle of primer extension at 72 C for 15 min (Harrington et al. 2001).

The PCR products were either purified using a QIAquick PCR Purification Kit (Qiagen INC., California, USA) or were digested with restriction enzyme. The PCR products were sequenced at the DNA Synthesis and Sequencing Facility at Iowa State University using the primers ITS1-F and ITS-4 for the ITS region or LROR and LR3 (5′–CCGTGTTTCAAGACGGG–3′) for the LSU.

The restriction enzyme HaeII (Gibco BRL. Inc., USA), which recognizes the base sequences GGCC and CCGG, was used to identify members of the *Ophiostoma piceae* complex (Harrington et al. 2001). Each unpurified PCR product (10 µl) was mixed with 2 µl of 10× buffer (supplied with the enzyme), 1 µl of restriction enzyme, and 7 µl of sterilized, distilled water. Digestion was allowed to proceed for 3 h at 37 C, and then restriction fragments were separated by electrophoresis for 3 h at 70 V in 1.6 % agarose gels with TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.0).
Comparison of rDNA sequences — Sequences of the LSU and ITS rDNA were compared with those of isolates of Ophiostomatales from our database and others available in the NCBI database using Megablast (BLASTN 2.2.22+).

Frequency comparisons.—The Chi-square test was used to compare the difference in presence of fungus species at different sites and from different beetle sexes. Chi-square was calculated as:

\[ X^2 = \sum \frac{(O_{ij} - E_{ij})^2}{E_{ij}}, \]

where \( X^2 \) represents the test statistic which approaches a \( X^2 \) distribution, \( O_{ij} \) represents an observed frequency, and \( E_{ij} \) represents an expected frequency. The Chi-square test was conducted at the statistical significance level of \( \alpha = 0.05 \).

RESULTS

Isolation of fungi from Hylurgus ligniperda.—In total, 118 adults of \( H. \ ligniperda \) (55 female and 63 male) were sampled for the presence of Ophiostomatales, and 114 individual isolates of Ophiostomatales were obtained on CSMA. Of the 118 beetles, 87 (74%) yielded at least one species of Ophiostomatales. Twenty-two percent of the beetles yielded more than one species of Ophiostomatales. On average, 0.96 species of Ophiostomatales were isolated from each adult sampled.

All isolated Ophiostomatales were grouped into 15 putative morphological species based on mycelial characteristics, teleomorphs, anamorphic states, and growth rate. Fourteen of the putative species were separated into four main groups based on their anamorphic states.
The first group contained *Pesotum* and *Sporothrix* synanamorphs, the second group contained *Leptographium* anamorphs, the third group contained only *Sporothrix* anamorphs, and the fourth group had *Hyalorhinocladiella* anamorphs. Another species produced no spores in culture.

Many of the species were similar to common *Ophiostoma* and *Leptographium* species (pigmented and macronematous conidiophores) reported from *H. ligniperda* from the Southern Hemisphere (Table I). The most common *Ophiostoma* species were distinguished by comparing rDNA sequences and morphological descriptions of Ophiostomatales (Aghayeva et al. 2004, 2005, Davidson 1945, 1953, Harrington et al. 2001, Hutchison and Reid 1988, Jacobs and Wingfield 2001, Ohtaka et al. 2006, Robinson-Jeffrey and Grinchenko 1964, Robinson-Jeffrey and Davidson 1968, Thwaites et al. 2005, Upadhyay 1981, Zhou et al. 2004 c). Overall, eight species were identified, but seven species could not be identified to species. One of the unidentified species is described here as new. The species are reported below in decreasing order of isolation frequency.

*Isolated species.—* *Ophiostoma ips* (Rumbold) Nannf.—Morphologically, *O. ips* was easily distinguished from other fungi by its brown colored mycelium and ascospores with rectangular sheaths. Many *O. ips* isolates produced perithecia and ascospores in culture within 3–4 wk after incubation. *Perithecia* dark brown to black, bases globose, (140–) 160–400 (–490) µm diameter, with straight or curved neck 180–1100 (–1300) µm long, and no ostiolar hyphae. *Ascospores* oblong, surrounded by a hyaline gelatinous sheath, appearing rectangular or square shaped, 2.4–4.8 × 1.3–2.5 µm. *Conidiophores* hyaline, (50–) 79–215 (–
240) μm long, conidiogenous cells without denticles. Conidia cylindrical to slightly lageniform, 2.3–5.8 (–7) × (1–) 1.5–2.5 (–4) μm. The ITS sequence of the O. ips isolates (GU129980) matched (594 of 594 nucleotides) those of O. ips isolates from different countries (e.g., AY546707, AY172021, and DQ539549).

Grosmannia galeiforme (Bakshi) Zipfel Z.W. de Beer & M.J. Winf. —All G. galeiforme isolates had light gray to dark brown mycelium and synnematous conidiophores (common) and rarely mononematous conidiophores. These isolates produced perithecia and ascospores on PTM within 6–8 wk when paired with mating testers. Perithecia bases (120–) 195–240 (–280) μm diameter, necks (510–) 600–710 μm long. Ascospores reniform with sheath, 1.8–4.3 × 1.0–2.4 μm. Synnemata 120–300 μm tall. Conidia cylindrical, (2–) 3.2–5 × 1–2.5 μm. The ITS sequences of all the G. galeiforme isolates (e.g., GU129981) matched (514 of 514 nucleotides) with the previously reported sequences of G. galeiforme from different countries (AY649770, DQ062979).

Ophiostoma piceae (Münch) H. & P. Sydow. —All O. piceae isolates had Pesotum and Sporothrix synanamorphs, and all tested isolates of O. piceae produced perithecia on PTM in 2 wk when paired with appropriate mating type testers. Perithecia with globose bases, 110–200 μm diameter, and straight necks 610–960 μm long. Ascospores reniform, (2–) 2.5–4.5 ×1.3–2.1 μm, accumulating on top of the perithecial neck. Synnemata dark brown, (160–) 210–1600 (–1930) μm long. Conidia cylindrical and ovoid to oblong, (2–) 2.8–5 (–6) × 1.0–2.3 μm. All isolates of O. piceae had the same HaeII restriction pattern, with fragments of 400, 200, 120 bp, as has been reported previously for this species (Harrington et al. 2001). Furthermore, ITS sequences of isolates of O. piceae (e.g., GU129988) matched
(575 of 575 nucleotides) the previously reported sequences of *O. piceae* from different countries (EF506934 and AF493249).

*Ophiostoma querci* (Georgévitch) Nannf. —All *O. querci* isolates produced a nut-like aroma and had a concentric ring pattern when grown on MEA (Harrington et al. 2001). Most isolates formed light-brown protoperithecia on MEA, and some isolates produced perithecia and ascospores on MEA by themselves within 10 d, and the perithecia had distinctive, long perithecial necks with glistening ascospore droplets, similar to *O. pluriannulatum* perithecia (Halmschlager et al. 1994, Harrington et al. 2001, Upadhyay 1981). All *O. querci* isolates had *Pesotum* and *Sporothrix* synanamorphs. *Perithecia* with globose bases 150–215 μm in diameter, necks straight or curved, 710–1420 μm long, with ostiolar hyphae. *Ascospores* reniform, 1.8–4.3 × 1.0–2.4 μm, accumulating on the tip of the neck. *Synnemata* dark brown to black, 290–830 (980) μm long. *Conidia* cylindrical or ovoid to oblong, 3–4.8 × 1–2.8 μm. All isolates of *O. querci* had the same *Hae*II restriction pattern, with fragments of 520 and 200 bp, consistent with previous reports for this species (Harrington et al. 2001). Furthermore, ITS sequences of the *O. querci* isolates (e.g., GU129989) matched (578 of 578 nucleotides) with the sequences of other previously-reported *O. querci* isolates (EF429089, FE506936, AF493246, and AY328520).

*Leptographium* sp. A. —All *Leptographium* sp. A isolates had identical ITS rDNA sequences (GU129991, GU129992, GU129993, GU129994, and GU129995) that were unique but similar to those of *G. aureum* (Rob.-Jeffr. & Davids.) Zipfel Z.W. de Beer & M.J. Winf., comb. (AY935606), *G. clavigerum* (Rob.-Jeffr. & Davids.) Zipfel Z.W. de Beer & M.J. Winf., comb. (AY263196), *G. robustum* (Rob.-Jefffr. & Davids.) Zipfel Z.W. de Beer &
M.J. Winf., comb. (AY263190, AY263189), *L. longiclavatum* Lee, Kim & Breuil (AY816686), *L. terebrantis* Barras & Perry (AY935607), and *L. pyrinum* Davidson (AY544621). Of the related species, *Leptographium* sp. A was morphologically most similar to *G. robustum* because of its globose conidia and granular material on the conidiophore stipes and hyphae, though *G. robustum* forms the granular material only on hyphae (Jacobs and Wingfield 2001, Robinson-Jeffery and Davidson 1968). The conidiophores of *Leptographium* sp. A are shorter than those of *G. robustum* and other *Leptographium* species. In addition, *G. robustum* forms a teleomorph in culture (Jacobs and Wingfield 2001, Robinson-Jeffrey et al. 1964), but *Leptographium* sp. A formed no perithecia in culture. *Leptographium* sp. A is described later as a new species.

*Sporothrix* sp. C.—Isolates of this unidentified *Sporothrix* formed colonies that were white to pale yellow on MEA. *Conidiophores* short, arising from thin septate hyphae. *Conidiogenous cells* with conspicuous denticles. *Conidia* ovoid to cylindrical with pointed ends, 2.1–4.5(–5.3) × 0.5–1.2 µm. The ITS sequence of this species (GU129986) was closely related to other unidentified *Sporothrix* species (de Beer et al. 2003) that were isolated from either soil or plant material (AY484468, 531 of 536 bases matching; and AF484471, 527 of 536 bases matching) and the recently described (de Meyer et al. 2008) *S. stylites* (EF 127884, 527 of 536 base pairs matching and EF127882, 530 of 536 bases matching).

*Grosmannia huntii* (Robins.-Jeff.) Zipfel Z.W. de Beer & M.J. Wingf. —Isolates of *G. huntii* failed to produce perithecia, even when paired with the mat-a mating tester of *G. huntii* from Chile. However, the *Leptographium* state of the California isolates was consistent with previous reports for *G. huntii* (Jacob et al. 1998, 2001, Robinson-Jeffrey and Grinchenko
Colonies dark greenish-olivaceous with smooth serpentine hyphae. Conidiophores light olivaceous, arising singly or in groups, 110–540 µm long, with 2–3 primary branches, 5–20 × 3–6 µm. Conidia obovoid with truncate bases and rounded apices, (3–) 4–5.4 (–6) × 1.5 (–3) µm. The ITS sequences of California isolates (e.g., GU129982) were identical to the ITS sequences of other *G. huntii* isolates, such as DQ 674362 and DQ674361 (both 588 of 588 bases matching).

*Leptographium serpens* (Goid.) Siem. —Morphological characters of isolates fell within the range reported for *L. serpens* (Jacobs and Wingfield 2001, Upadhyay 1981) and matched other isolates of *L. serpens* in the Iowa State University collection (C391 and C759 = CMW 290). Hyphae serpentine. Conidiophores with a large central metula, 3–5 primary metulae 8–20 × 4–10 µm, three or more secondary metulae on top of each primary metula. Conidia 3–5 × 1–2 µm. Originally *L. serpens* was described from *Pinus sylvestris* in Italy, and the description included a teleomorph (Goidanich 1936), but the only material available from the original collection is an isolate that does not produce perithecia (Harrington 1988). Thus, Harrington (1988) suggested using the anamorphic name *L. serpens* rather than *O. serpens*. Although it was not possible to obtain a full ITS sequence, the partial ITS-2 sequence of *L. serpens* that was obtained was similar to accessions AY554488 and AY707203 of *L. serpens* (81 bases matching). The LSU sequence (GU129998) of *L. serpens* matched closely with the sequences of other previously reported isolates of *L. serpens*, such as EU177471 (isolate C30, from the holotype) and AY707203 (both with 541 of 542 bases matching).
Ophiostoma floccosum Mathiesen. —The isolates of O. floccosum from California did not form perithecia and ascospores when paired with testers of opposite mating type, but they did produce typical Pesotum and Sporothrix anamorphic states. Synnemata red-brown with lateral knobs, 120–280 (–350) µm long, secondary synnemata frequently emanating from the conidial masses on the top of primary synnemata. Conidia cylindrical or fusiform with pointed base, 3–9 × 1–2.8 µm. These fungi had the HaeII restriction pattern typical of the ITS product (280 and 200 bp) of O. floccosum (Harrington et al. 2001).

Ophiostoma stenoceras (Robak) Melin & Nannf. —Colonies white to slightly yellowish, growth 85 mm diameter after 20–24 d at 25 C. Protoperithecia developed after 2 wk, and then perithecia and ascospores developed and matured slowly. Perithecia with bases (70–) 95–170 (–210) µm diameter, usually with a single long, straight neck 520–1500 (–1800) µm, but some with two short necks (280–) 300–420 µm, ostiolar hyphae present. Ascospores hyaline and curved or crescent shaped, small, 2–3(–3.9) × 1–1.5 µm. Conidiophores with distinct denticles on the conidiogenous cells. Conidia elongated or ellipsoidal with pointed ends, hyaline, (2–) 2.8–6.5 (–7.5) × (1–) 1.3–2 µm. The ITS sequence of the O. stenoceras isolates (GU129990) matched (538 of 539 bases) sequences of other O. stenoceras isolates, including accessions DQ539511, AY280492, and AF484476.

Sterile fungus. —This species did not produce perithecia or conidia on MEA, MYEA or PTM. Colonies white and fast-growing, filling an 85mm plate within a week at 25 C. Ophiostoma species without teleomorph or anamorph in culture are unusual, although Ohtaka et al. (2006) reported that O. rectangulosporium from Japan had a teleomorph state but not an anamorph state in culture. The sterile species had an ITS sequence (GU129987) that was
similar to sequences of *O. rectangulosporium* (AY242825, 532 of 550 bases matching; and GU124171, 529 of 550 bases matching).

*Sporothrix* species A and B (near *O. nigrocarpum* (Davids) de Hoog). — These two unidentified species differ slightly in their morphological characters. *Sporothrix* sp. A is similar to *O. nigrocarpum* isolates from the western pine beetle (C190, C210): Colonies grow slowly on MEA, 2.3–2.7 mm d−1 at 25 C, white to light yellowish, aerial mycelia with no concentric ring pattern. *Conidiogenous cells* usually short and slightly rough, with distinct denticles. *Conidia* clavate to ovoid with pointed end, small, 3.2–4.8 (–6.2) × 1–1.6 (–2.4) µm. In contrast, *Sporothrix* sp. B is very similar to isolates from southern pine beetle (C349 and C558): Colonies with abundant aerial mycelia and a concentric ring pattern. *Conidia* (4–5.6 (–6.4) × 1–1.8 (–2.5) µm) with large denticles 0.2–0.4 µm. The separation of *Sporothrix* sp. A and B was supported by comparison of ITS sequences. All isolates of *Sporothrix* sp. A had ITS sequences (GU129984) similar to those of western pine beetle isolates (DQ396788, 537 of 543 bases matching; and AF484452, 536 of 543 bases matching). The ITS sequences of isolates of *Sporothrix* sp. B (GU129985) matched those of southern pine beetle isolates C349 and C558, (541 of 541 bases matching) and were similar to those of *O. abietinum* Marmolejo & Hutin (DQ396788 and AF484453, 538 of 541 bases matching).

*Hyalorhinocladiella* sp. A. — Colonies white, with no aerial mycelium. *Hyphae* thick-walled, submerged under the medium. *Conidiophores* arising directly from the hyphae, lacking prominent denticles. *Conidia* broadly ellipsoidal to sub-cylindrical, with rounded ends, (0.9–) 1.2–1.9 × 3.2–5.2 µm. In the DNA sequence comparison, isolates of *Hyalorhinocladiella* sp. A were similar to those of *O. rectangulosporium* based on ITS
(GU129997, 528 of 549 bases matching DQ539538) and LSU (GU221905, 518 of 538 bases matching AB235158).

_Hyalorhinocladiella_ sp. B. —*Conidiophores* with large conidial masses. *Hyphae* hyaline, thick-walled, submerged in the medium. *Conidiophores* short, lacking distinct denticles at the point of conidium detachment. *Conidia* broadly ellipsoidal with both ends rounded, (7.5–) 13–18 × 4.5 (–5) µm. Isolates of _Hyalorhinocladiella_ sp. B had ITS sequences (GU129996) close to those of _O. piliferum_ (Fries) H. & P. Sydow (AY934516, 444 of 481 bases matching), _O. bicolor_ Davids. & Wells. (DQ268606, 442 of 481 bases matching), and _O. montium_ (Rumbold) von Arx. (AY546710, 454 of 481 bases matching) and LSU sequences (GU221906) close to those of _O. bicolor_ (DQ268605, 529 of 539 bases matching) and _Hyalorhinocladiella_ sp. (DQ268591, 526 of 539 bases matching).

TAXONOMY

**Leptographium tereforme** S.J. Kim & T.C. Harrin. _sp. nov._

Coloniae olivacea vel atro-viridae, ad 34 mm in 10 dies in 1.5% MEA. *Hyphae* brunnea, granulatae. *Conidiophorae* singulae aut aggregatae, (35–) 70 (–130) µm longae. *Stipae* brunnea vel atro-brunnea, granulatae, 1–4 septatae, (13–) 20–60 (–65) µm longae et (2–) 3–6 (–8) µm latae, con 0-5 metulae primae. *Conidia* hyalinae, eseptatae, oblongatae vel obovatae, apicibus rotundatis, 3.2–5 (–8) × 2.5–3.9 (–4.8) µm.

_Specimens examined._ USA. CALIFORNIA: Los Angeles County, La Cañada Flintridge, Descanso Gardens-, 34.21055°N, 118.20044°W, from female _Hylurgus ligniperda_,
26 Mar 2006, S. J. Kim, C2314 (Holotype, BPI879603, a dried culture of isolate C2314 = CBS125736).

_Etymology_. Latin _teres_ (rounded) and _forme_ (form, shape) referring to the rounded shape of the conidia.


_Colonies_ with optimal growth at 25 C on 1.5% MEA, attaining a diameter of 35 mm in 10 d, olive green to dark green or dark gray with age. No growth at 5 C and above 35 C. _Hyphae_ mostly submerged, covered by granular material (Fg 1. D). _Perithecia_ and ascospores absent. _Conidiophores_ occurring singly or in groups (3–4), micronematous, mononematous, (35–) 70 (–130) µm in length (Fg 1. A, B). _Rhizoid_-like structures absent. _Stipe_ brown to dark brown, becoming darker, covered with granular material on lower half to lower three-quarters, 1–4 septate, (13–) 20–60 (–65) µm long (from base to below primary branches), (2–) 3–6 (–7.5) µm wide below primary branches, apical cell not swollen, (3–) 4–7 (–10) µm wide at base, basal cell not swollen. _Conidiogenous apparatus_ (15–) 21–37 (–42) µm long excluding the conidial mass, with 1–4 series of cylindrical branches (Fg 1. C). _Primary branches_, if present, hyaline, smooth, cylindrical, (8–) 9–18 (–30) µm long.
Secondary branches absent or present, hyaline, (5–) 10 (–15) µm in long. Conidia hyaline, aseptate, oblong to slightly obovoid with round apices, 3–5 (–8) × 2.5–4.0 (–5) µm (Fig 1. E). Conidial droplet hyaline at first, becoming creamy-yellow, remaining the same color when dry.

Isolation frequencies. —Among the identified fungal species, O. ips had the highest frequency of occurrence. It was isolated from 31% of the 118 beetles sampled. Besides O. ips, other fungi with high frequencies of isolation included G. galeiforme (from 23%), O. piceae (9%), O. querci (8%) and L. tereforme (6%). In contrast, Sporothrix sp. C, G. huntii, L. serpens, O. floccosum, and Sporothrix sp. A were each isolated from less than 5% of the beetles (TABLE III). Each of the other species, including O. stenoceras, were only isolated from one beetle.

Chi-square tests showed no difference for any of the fungal species in frequency of isolations from male vs. female beetles at either site. However, there were differences in isolation frequencies of some species between the two major sites. At San Dimas, for instant, O. ips was the dominant species with a 62% frequency of occurrence from the 45 sampled beetles, and five different fungal species were isolated. At La Cañada Flintridge, O. ips was less common (from 11% of the beetles), and G. galeiforme was the dominant species with 25% frequency of occurrence, and O. piceae and O. querci were frequently isolated. In addition, L. tereforme and sterile fungus were found only at La Cañada Flintridge site (TABLE III).
DISCUSSION

Fifteen species of Ophiostomatales were isolated from the root-feeding bark beetle, *Hylurgus ligniperda*, in California. Most, but not all, of the isolated species had been reported previously in the USA, mostly as bluestain fungi on pine sapwood (Jacobs and Wingfield 2001, Harrington 1988, Harrington et al. 2001, Upadhyay 1981, Wingfield 1993). The frequency of occurrence of some of the fungi may indicate an intimate relationship with the bark beetle, but only *G. galeiforme* was isolated in significant frequency (20% or greater) at both sites. Fungal species diversity did not differ between male and female beetles, but the mycoflora between the two locations differed. At the La Cañada Flintridge site, 15 fungal species, including one new species, were isolated, but only six species were isolated from the beetles collected at the San Dimas site, where *O. ips* was isolated from 62% of the beetles.

*Grosmannia galeiforme* appeared to be a dominant fungus at both the California sites. This species is believed to be a European species (Bakshi 1951, Mathiesen–Käärik 1960), and has been associated with a wide range of European bark beetles, including *Orthotomicus erosus* (Wollaston), *Hyalstes angustatus* (Herbst), *Dryocoetes autographus* (Ratzeburg), and *Ips sexdentatus* (Boerner) (de Beer et al. 2003b, Romón et al. 2007, Zhou et al. 2004b, 2004c). *Grosmannia galeiforme* has been introduced into Chile, South Africa, and New Zealand with one or more of the above European bark beetles. For instance, in Chile and New Zealand, *G. galeiforme* was isolated from both *H. ligniperda* and *Hylastes ater* (Paykull) (Reay et al. 2002, 2005, 2006, Zhou et al. 2004a), and in South Africa, it was isolated from *H. ligniperda* (Zhou et al. 2001). Species closely related to *G. galeiforme* have been reported from North America (Zhou et al. 2004b, c), but *G. galeiforme* has not been
previously confirmed here. Thus, it is likely that *H. ligniperda* brought *G. galeiforme* with it when it was accidentally introduced to California. To date, there have been no reports of pathogenicity of *G. galeiforme* to pine, so the finding of this fungus in California may not be cause for concern.

*Ophiostoma ips* is known to be distributed worldwide as a bluestain fungus and associated with many conifer-feeding bark beetles (Reay et al. 2002, 2005, 2006, Romón et al. 2007, Zhou et al. 2001, 2004a, b), especially in association with *Ips* spp. on pines in North America (Mathre 1964, Rumbold 1931, Romón et al. 2007, Seifert 1993). The fungus has been introduced into several Southern Hemisphere countries through the accidental introduction of various coniferous bark beetles (Zhou et al. 2001, 2004a, 2006). These include the stem-infesting *Ips grandicollis* (Eichh.), which is native to North and Central America, and which has been introduced into Australia (Stone and Simpson 1990).

*Ophiostoma ips* was reported from New Zealand on *H. ligniperda*, but it was isolated from only 5% of the beetles sampled (Reay et al. 2006), and it was not reported in isolations from five adult *H. ligniperda* in Spain (Romón et al. 2007). In South Africa, it was isolated from 12.6% of the 199 sampled *H. ligniperda* adults (Zhou et al. 2001). In contrast, this species was reported with a high frequency of occurrence (isolated from 33.8% of 80 adult *H. ligniperda* in Chile (Zhou et al. 2004a). In USA, at the La Cañada Flintridge site, *O. ips* was isolated from only 11% of the beetles, though *O. ips* was isolated from 62% of the beetles sampled from the San Dimas site. The high frequency of occurrence of *O. ips* at the latter site may have been the cause of the relatively low incidence of other Ophiostomatales and the lower number of species isolated. Because *O. ips* is well known in North America and it
appears to be inconsistently associated with *H. ligniperda*, it is likely that the beetle acquired *O. ips* after its arrival to California.

*Ophiostoma piceae* and *O. querci* were commonly isolated from *H. ligniperda* collected at the La Cañada Flintridge site, and *O. floccosum* was isolated from two beetles at this site. However, these synnema-forming *Ophiostoma* species were not isolated from beetles collected at the San Dimas site. These species are not considered pathogens and are associated with blue-stain of conifers, although *O. querci* occurs primarily on hardwoods (Halmschlager et al. 1994, Harrington et al. 2001). *Ophiostoma floccosum* was originally described from Sweden, and it has been reported from Europe, Korea, New Zealand, and North America (de Beer et al. 2003b, Harrington et al. 2001) and has been associated with several bark beetle species, including *H. ligniperda* (Kirisits 2004, Reay et al. 2006, Romón et al. 2007, Zhou et al. 2006). In the past, the synnema-forming *Ophiostoma* species have been difficult to differentiate, and *O. querci* and *O. floccosum* were often treated as synonyms of *O. piceae* (Harrington et al. 2001). Thus, past studies with *H. ligniperda* may have considered *O. querci* and *O. floccosum* isolates as *O. piceae*. The association of *O. piceae* and *O. querci* with *H. ligniperda* was reported in South Africa (Zhou et al. 2001, 2006). No synnema-forming species was found associated with *H. ligniperda* in Chile (Zhou et al. 2004a). In Spain, Romón et al. (2007) isolated *O. piceae* and *O. querci* from several bark beetle species such as *Hylurgops palliatus* (Gyllenhal), *H. attenuates* Erichson, and *Tomicus piniperda* (Linnaeus), but not from *H. ligniperda*. In the New Zealand study (Reay et al. 2006), the synnema-forming species were distinguished, and *O. querci*, *O. floccosum*, and *O. setosum* were identified from *H. ligniperda*. However, these two species are not strict
associates of *H. ligniperda*, or even bark beetles, because both fungi often are encountered in the absence of bark beetle activity (Harrington et al. 2001, Reay et al. 2002, 2006, Thwaites et al. 2005). Thus, the synnema-forming species of *Ophiostoma* are common, but not consistent, associates of *H. ligniperda*, and they are not specific to this beetle (Harrington et al. 2001).

*Grosmannia huntii* was first described from pine infested with a *Dendroctonus* sp. in Canada (Robinson-Jeffrey and Grinchenko 1964). *Grosmannia huntii* is often confused with *G. piceaperdum* (Rumbold) Goid. because similar insect vectors (pine- and spruce-infesting insects) and similar morphology (*Leptographium* anamorphs and hat-shaped ascospores) (Jacobs et al. 1998). However, Jacobs et al. (1998) separated *G. huntii* and *G. piceaperdum* by morphology and sexual compatibility. *G. huntii* has been associated with many different bark beetle species on *Pinus* and *Picea* spp. in several countries: *D. ponderosae* (Hopkins), *Ips pini* (Say), and *Hylastes macer* (Le Conte) in the USA. (Harrington 1988, Jacobs and Wingfield 2001), *Tomicus piniperda* in Europe (Gibbs and Inman 1991), and *H. ligniperda* in New Zealand (Reay et al. 2006). In New Zealand (Reay et al. 2006), *G. huntii* was the most commonly encountered species and was isolated from 83% of the *H. ligniperda* adults sampled. Thus, the association between *G. huntii* and *H. ligniperda* found in this study was not surprising, but the frequency of this species from California beetles was low.

*Leptographium serpens* has been associated with root diseases of *P. radiata*, *P. pinaster* and other *Pinus* spp. in the southeastern U. S., and it has been isolated from *H. angustatus* and *H. ligniperda* in South Africa (Harrington 1988, Jacobs and Wingfield 2001, Wingfield et al. 1988, Zhou et al. 2001). It has been associated with *H. angustatus* and *H.*
*ligniperda* or *Hylastes* spp., which are root-feeding bark beetles native to Europe (Reay et al. 2002, 2005, 2006, Thwaites et al. 2005, Zhou et al. 2001, 2002, 2004a). For example, in South Africa (Zhou et al. 2001) 45% of the *Ophiostoma* isolates from *H. angustatus* and 21% of the isolates from *H. ligniperda* were identified as *L. serpens*. In California, *L. serpens* was isolated from *H. ligniperda* at both study sites, though in low frequency. Because *L. serpens* has not been reported from western North America but it has been commonly associated with European bark beetle species such as *H. ligniperda*, it is possible that this beetle brought the fungus with it to California. *Leptographium serpens* has not been considered a serious pathogen to *Pinus* spp. (Wingfield et al. 1988, Zhou et al. 2002) or as a contributor to loblolly pine decline (Eckhardt et al. 2007). In the southeastern USA, however, *L. serpens* was weakly pathogenic to wounded loblolly pine seedlings under low soil moisture conditions (Matustick et al. 2008). Thus, the presence of *L. serpens* in California needs to be monitored further, and pathogenicity tests may be warranted.

The new species, *Leptographium tereforme*, was isolated from 6% of the *H. ligniperda*, but only at the La Cañada Flintridge site. Thus, *H. ligniperda* may have acquired the fungus from another bark beetle. This fungus is distinguished from related *Leptographium* species such as *G. aureum*, *G. clavigerum*, *G. robustum*, *L. longiclavatum*, *L. terebrantis*, and *L. pyrimum* by rDNA sequence comparisons and morphology. The rDNA sequences of *L. tereforme* were closest to those of *L. terebrantis*, *L. wingfieldii* Morelet, *L. truncatum* (M. J. Wingf. & Marasas) Wingfied, *L. lundbergii*, and *L. guttulatum* M.J. Wingf. & Jacobs. Morphologically, *L. tereforme* is easily distinguished from these species by its
globose conidia, short conidiophores, and granular material covering half or up to three quarters of the conidiophore stipe.

Although *L. tereforme* has not been previously reported, it is possible that reports of *L. guttulatum*, *L. lundbergii*, or *L. truncatum* from *H. ligniperda* in Spain, South Africa, and New Zealand (Romón et al. 2007, Zhou et al. 2001, 2004a) were actually *L. tereforme*. *Leptographium guttulatum* has been associated with bark beetles in the genera *Dryocoetes*, *Hylates*, *Hylurgops*, and *Tomicus* (Jacobs et al. 2001, Romón et al. 2007, Wingfield and Gibbs 1991). *Leptographium lundbergii* was only reported from South Africa (Zhou et al. 2001), but the taxonomic of this species has been confused in the past. *L. lundbergii* was erroneously reduced to synonymy with *L. truncatum* (Jacobs and Wingfield 2001, Strydom et al. 1997), even though Zambino and Harrington (1992) provided evidence that *L. lundbergii* and *L. truncatum* represent discrete species using isozyme data. Therefore, it is possible that Zhou et al. (2001) may have misidentified *L. truncatum* or *L. tereforme* isolates as *L. lundbergii*. Jacobs et al. (2005) reported that *L. truncatum* and *L. lundbergii* are distinct by DNA sequence comparisons and morphology. *Leptographium truncatum* was rarely isolated from *H. ligniperda* in New Zealand (Reay et al. 2006).

The other species reported from California were isolated from only one or two beetles. *Ophiostoma stenoceras* has been commonly isolated from several bark beetle species (Kirisits 2004, Romón et al. 2007, Zhou et al. 2001, 2006), but it is not generally considered to be a strict associate of bark beetles. *Sporothrix* sp. A, B, and C, and the two unidentified *Hyalorhinocladiella* species may be conspecific with unidentified species from *H. ligniperda*
in New Zealand (Reay et al. 2006), Spain (Romón et al. 2007), or South Africa (Zhou et al. 2001).

A sterile fungus from *H. ligniperda* in California was placed near *O. rectangulosporium* based on rDNA sequences. Like the unidentified sterile fungus, *O. rectangulosporium* does not form conidia in culture (Ohtaka et al. 2006), but it has been associated with bark beetles such as *Cryphalus montanus* Nobuchi, *Polygraphus proximus* Blandford, and *Dryocoetes striatus* Eggers infesting *Abies* species (Yamaoka et al. 2004). Romón et al. (2007) reported *O. rectangulosporium* from *H. ligniperda* in Spain, but the rDNA sequence of the Spanish isolates was not similar to those of the Japanese isolates.

*Leptographium procerum* was reported as an associate of *H. ligniperda* from New Zealand (Reay et al. 2006), but it was not found associated with *H. ligniperda* in California. *L. procerum* has been commonly associated with root and root collar insects around the world (Jacobs and Wingfield 2001).

Another species, *Ceratocystiopsis minuta* was not isolated from *H. ligniperda* in California, though it was reported from Chile and South Africa in association with *H. ligniperda* (Zhou et al. 2001). There has been confusion about the taxonomy of members of the genus *Ceratocystiopsis* (Plattner et al. 2009), but none of the rDNA sequences of the California isolates from *H. ligniperda* were close to those of any *Ceratocystiopsis* species.

Most of the species isolated from *H. ligniperda* in California are common bark beetle associates in the USA and around the world, and many of the species had been associated with *H. ligniperda* in the Southern Hemisphere. It is likely, however, that the beetle brought only a few species, perhaps only *G. galeiforme* and *L. serpens*, with it when introduced to
California, and the other fungi were acquired in the USA and are not consistent associates of the beetle.

ACKNOWLEDGMENTS

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Bakshi BK. 1951. Studies on four species of *Ceratocystis*, with a discussion on fungi causing sapstain in Britain. Mycol Pap 35:1–16.


58


**TABLE I.** Species of *Ophiostoma*, *Grosmannia* and related anamorph genera associated with *Hylurgus ligniperda* in earlier studies.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ceratocystiopsis minuta</em></td>
<td>Chile</td>
<td>Zhou et al. 2004a</td>
</tr>
<tr>
<td></td>
<td>South Africa</td>
<td>Zhou et al. 2001</td>
</tr>
<tr>
<td><em>Ophiostoma floccosum</em></td>
<td>New Zealand</td>
<td>Reay et al. 2006</td>
</tr>
<tr>
<td></td>
<td>South Africa</td>
<td>Zhou et al. 2006</td>
</tr>
<tr>
<td><em>Grosmannia galeiforme</em></td>
<td>Chile</td>
<td>Zhou et al. 2004a</td>
</tr>
<tr>
<td></td>
<td>New Zealand</td>
<td>Reay et al. 2006</td>
</tr>
<tr>
<td></td>
<td>South Africa</td>
<td>Zhou et al. 2001</td>
</tr>
<tr>
<td><em>Grosmannia huntii</em></td>
<td>New Zealand</td>
<td>Reay et al. 2006</td>
</tr>
<tr>
<td><em>Ophiostoma ips</em></td>
<td>Chile</td>
<td>Zhou et al. 2004a</td>
</tr>
<tr>
<td></td>
<td>New Zealand</td>
<td>Reay et al. 2006</td>
</tr>
<tr>
<td></td>
<td>South Africa</td>
<td>Zhou et al. 2001</td>
</tr>
<tr>
<td><em>Ophiostoma piceae</em></td>
<td>South Africa</td>
<td>Zhou et al. 2001</td>
</tr>
<tr>
<td><em>Ophiostoma pluriannulatum</em></td>
<td>South Africa</td>
<td>Zhou et al. 2001, 2006</td>
</tr>
<tr>
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<td>New Zealand</td>
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</tr>
<tr>
<td></td>
<td>South Africa</td>
<td>Zhou et al. 2006</td>
</tr>
<tr>
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<td>Reay et al. 2006</td>
</tr>
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<td>South Africa</td>
<td>Zhou et al. 2001</td>
</tr>
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<td>Spain</td>
<td>Romón et al. 2007</td>
</tr>
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<td>South Africa</td>
<td>Zhou et al. 2001</td>
</tr>
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<td>New Zealand</td>
<td>Reay et al. 2006</td>
</tr>
<tr>
<td><em>Leptographium serpens</em></td>
<td>South Africa</td>
<td>Wingfield et al. 1988</td>
</tr>
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<td></td>
<td></td>
<td>Zhou et al. 2001</td>
</tr>
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<td>Reay et al. 2006</td>
</tr>
<tr>
<td></td>
<td>South Africa</td>
<td>Wingfield et al. 1988</td>
</tr>
<tr>
<td><em>Pesotum spp.</em></td>
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<td>Zhou et al. 2001</td>
</tr>
<tr>
<td><em>Hyalorhinocladiella sp.</em></td>
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<td>Zhou et al. 2001</td>
</tr>
<tr>
<td><em>Sporothrix sp.</em></td>
<td>South Africa</td>
<td>Zhou et al. 2001</td>
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</tbody>
</table>
TABLE II. Number of male and female adults of *Hylurgus ligniperda* collected live from galleries in the phloem of cut stem-sections of pines (*P. halepensis* and other *Pinus* spp.) at two locations in California.

<table>
<thead>
<tr>
<th>City</th>
<th>Detail location</th>
<th>Pine species</th>
<th>Number of sampled <em>H. ligniperda</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Female</td>
</tr>
<tr>
<td>Descanso Gardens</td>
<td>Maintenance building</td>
<td><em>P. halepensis</em></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Parking lot</td>
<td><em>Pinus</em> spp.</td>
<td>28</td>
</tr>
<tr>
<td>Bonelli Park</td>
<td>Dam</td>
<td><em>P. halepensis</em></td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Golf Course</td>
<td><em>Pinus</em> spp.</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Via Verde Dr.</td>
<td><em>Pinus</em> spp.</td>
<td>0</td>
</tr>
<tr>
<td>Species</td>
<td>La Cañada Flintridge</td>
<td>San Dimas</td>
<td>GenBank Accession No.</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------------------</td>
<td>-----------</td>
<td>-----------------------</td>
</tr>
<tr>
<td></td>
<td>ITS</td>
<td>LSU</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma ips</em></td>
<td>8 (11%)</td>
<td>28 (62%)</td>
<td>GU129980</td>
</tr>
<tr>
<td><em>Grosmannia galeiforme</em></td>
<td>18 (24.6%)</td>
<td>9 (20%)</td>
<td>GU129981 GU221908</td>
</tr>
<tr>
<td><em>Ophiostoma piceae</em></td>
<td>11 (15%)</td>
<td>0</td>
<td>GU129988</td>
</tr>
<tr>
<td><em>Ophiostoma querci</em></td>
<td>10 (13.7%)</td>
<td>0</td>
<td>GU129989</td>
</tr>
<tr>
<td><em>Leptographium tereforme</em></td>
<td>7 (9.6%)</td>
<td>0</td>
<td>GU129994</td>
</tr>
<tr>
<td><em>Sporothrix</em> sp. C</td>
<td>4 (5.5%)</td>
<td>1 (2.2%)</td>
<td>GU129986</td>
</tr>
<tr>
<td><em>Grosmannia huntii</em></td>
<td>2 (2.7%)</td>
<td>3 (6.7%)</td>
<td>GU129982 GU129999</td>
</tr>
<tr>
<td><em>Leptographium serpens</em></td>
<td>2 (2.7%)</td>
<td>2 (4.4%)</td>
<td>GU129983 GU129998</td>
</tr>
<tr>
<td><em>Ophiostoma floccosum</em></td>
<td>2 (2.7%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Sporothrix</em> sp. A</td>
<td>1 (1.4%)</td>
<td>1 (2.2%)</td>
<td>GU129984 GU221907</td>
</tr>
<tr>
<td><em>Ophiostoma stenoceras</em></td>
<td>1 (1.4%)</td>
<td>0</td>
<td>GU129990</td>
</tr>
<tr>
<td><em>Sporothrix</em> sp. B</td>
<td>1 (1.4%)</td>
<td>0</td>
<td>GU129985</td>
</tr>
<tr>
<td><em>Hyalorhinocladiella</em> sp. A</td>
<td>1 (1.4%)</td>
<td>0</td>
<td>GU129997 GU221905</td>
</tr>
<tr>
<td><em>Hyalorhinocladiella</em> sp. B</td>
<td>1 (1.4%)</td>
<td>0</td>
<td>GU129996 GU221906</td>
</tr>
<tr>
<td>Sterile fungus</td>
<td>1 (1.4%)</td>
<td>0</td>
<td>GU129987</td>
</tr>
<tr>
<td>No fungus isolated</td>
<td>24 (32.9%)</td>
<td>8 (17.8%)</td>
<td></td>
</tr>
</tbody>
</table>

1 GeneBank accession numbers correspond to sequences of representative isolates of each species.
TABLE IV. Number and frequency of isolation of *Ophiostoma* and related species from adult *Hylurgus ligniperda* collected from New Zealand and USA (California, this study).

<table>
<thead>
<tr>
<th>Species</th>
<th>Country</th>
<th>New Zealand</th>
<th>USA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ceratocystis minuta</em></td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Grosmannia galeiforme</em></td>
<td>115 (95.8%)</td>
<td>27 (22.9%)</td>
<td></td>
</tr>
<tr>
<td><em>Grosmannia huntii</em></td>
<td>100 (83.3%)</td>
<td>5 (4.2%)</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma floccosum</em></td>
<td>6 (5%)</td>
<td>2 (1.9%)</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma ips</em></td>
<td>6 (5%)</td>
<td>36 (30.5%)</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma piceae</em></td>
<td>0</td>
<td>11 (9.2%)</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma querci</em></td>
<td>2 (1.7%)</td>
<td>10 (8.5%)</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma setosum</em></td>
<td>7 (5.8%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma stenoceras</em></td>
<td>0</td>
<td>1 (0.8%)</td>
<td></td>
</tr>
<tr>
<td><em>Leptographium procerum</em></td>
<td>7 (5.8%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Leptographium serpens</em></td>
<td>0</td>
<td>4 (3.4%)</td>
<td></td>
</tr>
<tr>
<td><em>Leptographium tereforme</em></td>
<td>0</td>
<td>7 (5.9%)</td>
<td></td>
</tr>
<tr>
<td><em>Leptographium truncatum</em></td>
<td>1 (0.8%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Sterile fungus</td>
<td>0</td>
<td>1 (0.8%)</td>
<td></td>
</tr>
<tr>
<td><em>Hyalorhinocladiella</em> spp.</td>
<td>0</td>
<td>2 (1.7%)</td>
<td></td>
</tr>
<tr>
<td><em>Sporothrix</em> spp.</td>
<td>0</td>
<td>8 (6.8%)</td>
<td></td>
</tr>
<tr>
<td>No fungus isolated</td>
<td>Not known</td>
<td>32 (27.1%)</td>
<td></td>
</tr>
</tbody>
</table>

1 Reay et al.

2 n = the total number of sampled beetles.
FIGURE 1. Conidiophores, conidia and hyphae of *Leptographium tereforme*. A-C.

CHAPTER 3. *Ophiostoma ips* and related fungi isolated from a European bark beetle, *Orthotomicus erosus*, in California

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**Abstract:** The pine-infesting bark beetle *Orthotomicus erosus* is native to the Mediterranean and Middle East but was detected in southern California in 2004. This bark beetle has a broad host range and is a common vector of Ophiostomatales, some of which are tree pathogens or causal agents of blue stain in conifer sapwood. In this study, *Ophiostoma* and related genera were isolated on a cycloheximide-amended medium from 202 adult *O. erosus* specimens collected from infested pines (*Pinus brutia*, *P. halepensis*, and *P. pinea*) at four different sites in southern California. In total, five numbers of Ophiostomatales were isolated from *O. erosus*. The most frequently encountered species was *O. ips*, which was isolated from 85% of the beetles. Beside of *O. ips*, a sterile fungus was isolated from 16% of the beetles, and two *Sporothrix* species and a species of *Hyalorhinocladiella* were isolated from fewer then 3% of the beetles. *Ophiosotma ips* was already well-known in the USA, and has been found in association with *O. erosus* in other countries.

**Key words:** pine-infesting bark beetle, Ophiostomatales
INTRODUCTION

Orthotomicus erosus (Wollaston) (Coleoptera: Scolytidae) infests stems of Pinus spp. and is native to central and southern Europe and countries around the Mediterranean Sea. It has been introduced accidently to Chile, China, South Africa, and Swaziland (Haack 2001, Lee et al. 2008), and it was recently introduced into southern California, USA (Haack 2004). This bark beetle, called the Mediterranean pine engraver, has a broad host range, including several different Pinus spp. and other non-pine conifers such as cedar (Cedrus spp.), Douglas-fir (Pseudotsuga menziesii), and fir (Abies) (Lee et al. 2008, Seybold and Downing 2009).

Orthotomicus erosus was first detected in the USA in May 2004 in Fresno, California, during a survey by the California Department of Food and Agriculture (Seybold and Downing 2009), and it has been the second most frequently intercepted bark beetle species at US ports (Haack 2001). Thus, further introductions are likely.

Like many other bark beetles, O. erosus is a secondary pest. Although some researchers have reported that O. erosus may directly injure pine or other coniferous trees (Baylis et al. 1986, Jiang et al. 1992), it commonly infests stressed trees, recently fallen trees, or broken branches (Haack 2001, 2004, Lee et al. 2008, Seybold and Downing 2009).


Blue-stain fungi include morphologically related but phylogenetically distinct genera of Microascales (i.e., Ceratocystis) and Ophiostomatales (close to the Diaporthales) (Zipfel 2006). Although some conifer bark beetles transmit Ceratocystis species, most bluestain
fungi associated with bark beetles belong in the Ophiostomatales (Harrington 2005), especially members of the genera *Ophiostoma*, *Grosmannia*, and *Ceratocystiopsis* and their associated anamorph genera *Leptographium*, *Pesotum*, *Hyalorhinocladiella* and *Sporothrix*.

Species of Ophiostomatales produce sticky ascospores at the tips of perithecia and/or wet droplets of conidia on top of conidiomata, and the sticky spores masses are transferred tree to tree by bark beetles (Harrington 2005, Pain et al. 1997, Six 2003).

Previous researchers isolated *O. Ips*, *O. pluriannullatum* (Hedgc.) Syd. & P. Syd., *O. stenoceras* (Robak) Nannf., *L. guttulatum* M.J. Wingf. & Jacobs, *L. lundbergii* Lagerb. & Melin, *L. serpens* (Gold.) Siem., and *Pesotum* spp. from *O. erosus* in South Africa (Zhou et al. 2001), Spain (Romón et al. 2007), and Tunisia (Ben Jamaa et al. 2007) (Table V). No research has examined the relationship between *O. erosus* and Ophiostomatales in the USA.

This study examines the relationship between *O. erosus* and Ophiostomatales in California, where the beetle has become established and could have introduced pathogenic fungi or bluestain fungi from other continents. Beetles were excavated from infested bolts, and fungi were isolated on cycloheximide-amended media, which is semi-selective for the growth of *Ophiostoma* and related genera (Harrington 1981).

**MATERIALS AND METHODS**

*Isolation of fungi.* —In total, 202 adults *O. erosus*, 101 beetles of each gender, were excavated from naturally-infested bolts at four different sites in California on Dec 2005 and Mar 2006 (Table VI). Seventy six of the darkened adult *O. erosus* were collected from *P. pinea* and *P. halepensis* at the Bakersfield site (Kern County, 35°20'25"N, 199°06'38"W), 50
of the mixed dark and teneral *O. erosus* were collected from *P. brutia, P. halepensis,* and *P. pinea* at Fresno site (Fresno Country, 36°46′37″N, 119°42′35″W), 26 of the mixed dark and teneral *O. erosus* were collected from *P. halepensis* and *P. pinea* at Poterville site (Tulare County, 36°01′11″N, 119°05′59″W), and 50 of the mixed dark and teneral *O. erosus* were collected from *P. halepensis* and *P. pinea* at Visalia site (Tulare County, 36°19′15″N, 119°22′31″W). After collection, adults were individually stored in 1.5 ml microcentrifuge tubes and placed on ice. Sex was determined based on the posterior spines, and adults were individually crushed with a flame-sterilized tweezer. Each adult was stored in a microcentrifuge tube and sealed before sending to Iowa, where each beetle was stored in a refrigerator until isolations were attempted. The beetles were cut into four parts (head, prothorax, mesothorax and abdomen) and placed into cycloheximide–streptomycin malt agar (CSMA, 10 g malt extract and 15 g agar per 1L dH₂O, with 200 mg cycloheximide and 100 mg streptomycin added after autoclaving). Cycloheximide is semi-selective for *Ophiostoma* spp. and related genera (Harrington 1981). Cultures were incubated at room temperature until mycelium became visible on the medium, and then conidial masses or the edge of the mycelium were transferred to fresh plates of 1.5% malt extract agar (MEA, 15 g malt extract and 20 g agar per 1L dH₂O). Representative isolates of each putative species were stored at −80 C in the culture collection at Iowa State University.

*Fungal morphology.* —For morphological investigation, stored isolates were grown at room temperature on 1.5% MEA or on pine twig media (PTM, one piece of autoclaved pine twig added to molten, autoclaved 1.5% MEA) for mating experiments (Harrington 1992). Each
isolate was examined by light microscopy with differential interference (DIC) by Normarsky (Olympus BH-12 with Kodak DS 120). To identify species, morphology of each isolate was compared with described Ophiostomatales.

**DNA extraction.** — For DNA extraction, each isolate was grown at room temperature for 7 d on malt yeast extract agar (MYEA, 15 g malt extract, 2 g yeast and 20 g agar per 1 L dH₂O). The DNA was extracted from mycelium by an ‘Eppendorf DNA extraction method,’ which was modified from DeScenzo and Harrington (1994). Approximately 50 mg of wet mycelium was ground to a fine powder using a mortar and liquid nitrogen in a 1.5 ml microcentrifuge tube and suspended in 500 µl DNA extraction buffer (200 mM Tris-HCl pH 8, 25 mM EDTA pH 8, 150 mM NaCl, 0.5% SDS). After incubating at 65°C for 1 h, 200 µl of potassium acetate was added, and the mixture was placed into ice for 20 min and then centrifuged at 13,200 rpm for 15 min. The supernatant was placed into a new 1.5 ml microcentrifuge tube. An aliquot of cold isopropanol (406 µl) was added to the supernatant and incubated at −20°C for 1 h to precipitate the DNA. After centrifugation at 13,200 rpm for 10 min, the supernatant was discarded. The pellet was washed with 1.5ml of 70% ethanol, incubated at −20°C for 1 h, and centrifuged again as before. Finally the supernatant was discarded, the DNA pellet was allowed to air dry for 1 h, and it was resuspended in 100 µl of dH₂O to use for PCR amplification.

**DNA sequencing and rDNA sequence comparison.** — Attempts were made to amplify the 26S gene (nuclear large subunit, LSU) of the rDNA and the internal transcribed spacer (ITS)
regions of representative isolates of each putative species. The LSU region was amplified using the primers LROR (5′–ACCCGCTGAACATGAAGC–3′) and LR5 (5′–TCCTGAGGAACTTCG–3′) (Vilgalys and Hester 1990), and the ITS and the 5.8S gene were amplified using the universal fungal primers ITS1-F (5′–CTTGGTCATTAGAGAAGTAA–3′) (Gardes and Bruns 1993) and ITS-4 (5′–TCCTCCGCTTTATGATATATGC–3′) (White et al. 1990). The template DNA was amplified in a 50 µl reaction volume containing 1.25 units Takara Ex Taq Polymerase, 1X PCR reaction buffer, 200 µM dNTPs, 5% (V/V) DMSO, and 0.25 µM of each primer (Takara Mirus Bio. Japan). Cycling conditions were as follows: initial denaturation at 94 C for 2 min, then 35 cycles of annealing at 49 C for LSU and at 54 C for ITS for 35 s, and primer extension at 72 C for 2 min, followed by one final cycle of primer extension at 72 C for 15 min (Harrington et al. 2001).

The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen INC., California, USA). Each PCR product was sequenced using the primers ITS1-F/ ITS-4 for the ITS region and primers LROR/ LR3 (5′–CCGTGTTTCAAGACG–3′) for the LSU region at the DNA Synthesis and Sequencing Facility at Iowa State University.

The LSU and ITS rDNA sequences were aligned using BioEdit version 5.0.6 (North Carolina State University). Sequences of the LSU and ITS rDNA were compared with those of isolates of Ophiostoma from our database and others available in the NCBI database using Megablast (BLASTN 2.2.22 +).
Frequency comparisons. — The Chi-square test was used to compare the difference in incidence of fungal species at different sites and from different beetle sexes (male vs. female). Chi-square was calculated as:

\[ X^2 = \sum ((O_{ij} - E_{ij})^2 / E_{ij}), \]

where \( X^2 \) represents the test statistic which approaches a \( X^2 \) distribution, \( O_{ij} \) represents an observed frequency, and \( E_{ij} \) represents an expected frequency. The Chi-square test was conducted at statistical significance level \( \alpha = 0.05 \).

RESULTS

Isolation of fungi. — In total, 202 adults of \( O. erosus \) collected from four different sites in California were used for isolations, and 219 individual isolates of Ophiostomatales were obtained on CSMA. Among the 202 adults beetles sampled, 75% yielded at least one species of Ophiostomatales, and 18% yielded more than one species of Ophiostomatales. On average, 1.1 species of Ophiostomatales were isolated from each adult beetle.

All isolated Ophiostomatales were grouped into three groups based on mycelia characteristics, teleomorphs, and anamorphs. The first group contained more than 80% of the isolates, which produced brown colored mycelium and perithecia, developed deeply in the agar. The second group contained more than 10% of the isolates and did not produce conidia or ascospores. The third group contained the remaining isolates, which produced \( Sporothrix \) or \( Hyalorhinocladiella \) anamorphs. The isolated species were distinguished by comparing rDNA sequences and morphological descriptions of Ophiostomatales (Aghayeva et al. 2004,
de Beer et al. 2003, Davidson 1945, Marmolejo and Butin 1990, Ohtaka et al. 2002, 2006, Upadhyay 1981), but only one of the species was positively identified.

Isolated species. — *Ophiostoma ips* (Rumbold) Nannf. — *Ophiostoma ips* was the most frequently isolated species and was easily distinguished by ascospores with rectangular sheaths and brown mycelium. Most, but not all, *O. ips* isolates produced perithecia and ascospores in culture within 3–4 wk after incubation or paring with mating testers (C994 mat-a and C2308 mat-b from New Zealand). *Perithecia* dark brown to black, bases globose, 120–360 (–400) μm in diameter, with straight necks, 800–1240 μm long, and no ostiolar hyphae. *Ascospores* oblong, surrounded by a hyaline gelatinous sheath, appearing rectangular or square shaped, 1.2–2.9 × 3–4.5 μm. *Conidiophores* phialidic, 65–210 (–245) μm long, conidiogenous cells without denticles. *Conidia* ellipsoidal to ovoid, 1–2.8 (–3.4) × (2.8–) 3.2–6 (–7.2) μm. The LSU sequence of an *O. ips* isolate from *Orthotomicus erosus* (GU393353) matched (538 of 539 nucleotides) those of other *O. ips* isolates from *Dendroctonus ponderosae* Hopkins (AY194940) and from infected pines (AY172021) in Canada.

Sterile fungus. — This species did not produce perithecia or conidia on MEA, MYEA, or PTM. *Colonies* white that changed to creamy yellowish color with age and fast-growing, filling an 85mm plate within 7 d at 25C. *Ophiostoma* species without teleomorph or anamorph in culture are unusual, although Ohtaka et al. (2006) reported that *O. rectangulosporium* had a teleomorph state and no conidial state in culture. The ITS sequence of an isolate of the sterile fungus (GU393357) was similar to the ITS sequence of *O.*
rectangulosporium: AB242825 (at 524 of 551 bases) of a isolate from *Abis veitchii* in Japan, GU134171 (at 522 of 551 bases) of a isolate from *Ips subelongatus* Motsch in Japan, and EU785451 (at 518 of 551 bases) from a isolate from *D. valens* LeConte in China.

*Sporothrix* sp. C. —This unidentified *Sporothrix* species formed white colonies on MEA. *Colonies* white and later becoming grayish in culture and slow-growing, filling a 85mm plate within 3 wk at 25C. *Conidiophores* short, arising from the hyphae. Conidiogenous cells with short denticles 0.2–0.5 µm long. *Conidia* of two different shapes: one cylindrical with pointed tip, 2–3 (–3.8) × (3.8–) 5 (–9) µm, and the other ellipsoidal with rounded tip, 1.2–2.3 × 5–6.2 µm. The ITS sequence of an isolate of *Sporothrix* sp. C (GU393352) was similar to those of other *Sporothrix* spp., including *S. schenckii* (AF484468 at 531 of 536 bases matching), *S. stylites* (EF127884 at 527 of 536 bases matching), and an unidentified *Ophiostoma* species from South Africa (EU660452 at 518 of 536 bases matching) (Roets et al. 2009). The *Sporothrix* sp. C isolates from *O. erosus* had the same ITS sequence as *Sporothrix* sp. C isolates from *H. ligniperda* (Chapter 2), although the shape and size of conidia differed slightly.

*Sporothrix* sp. B (near *O. nigrocarpum* (Davids) de Hoog). —Morphologically, isolates of this species are identical to isolates of *Sporothrix* sp. B from *H. ligniperda* (Chapter 2) and similar to *O. nigrocarpum* isolates from the southern pine beetle, *Dendroctonus frontalis* (e.g., isolates C349 and C558). Isolates of *Sporothrix* sp. B produce white to creamy yellowish mycelium on MEA. *Colonies* slow-growing, filling a 85mm plate with 3 wk at 25C, aerial mycelium with a concentric ring pattern. *Conidiogenous cells* short, with tiny distinct denticles. *Conidia* ellipsoidal with rounded ends, small, (0.9–) 1.2–1.8 ×
The ITS sequence of this species (GU393350) differed slightly from the ITS sequence of Sporothrix sp. B from H. ligniperda (GU129985). Sporothrix sp. B from O. erosus had ITS sequences that were the same (541 of 541 nucleotides) as previous reported sequences of species such as O. abietinum Marmolejo & Hutin (DQ396788) and Sporothrix sp (AY280487), and similar to those of O. nigrocarpum (DQ 118420 and AY672915 at 538 of 541 bases matching).

Hyalorhinocladiella sp. C. —Colonies light brown to dark brown, diffusion of pigment into the agar. Hyphae covered with granular material. Conidiophores short, 10–14 (–20) μm long, lacking prominent denticles. Conidia ellipsoid or clavate shape with rounded ends, (1–) 1.2–2.6 (–3.1) × 4–6 (–7.2) μm. The ITS sequence of Hyalorhinocladiella sp. C (GU393355) was somewhat similar to that of S. inflata de Hoog (AY495433 at 453 of 521 bases, and AY495432 at 452 of 521 bases) as well as O. sejunctum M. Vilarreal, Arenal, V. Rubio & H. de Troya (AY934519, at 451 of 521 bases matching). In contrast, the results of the LSU sequence comparison (GU393354) indicated that Hyalorhinocladiella sp. C was related to an isolate of S. schenckii (AB363791, at 522 of 541 bases matching) and Fragosphaeria purpurea Shear (AF096191, at 519 of 541 bases matching).

Hyalorhinocladiella sp. C is distinct from the Hyalorhinocladiella spp. associated with H. ligniperda (Chapter 2) in morphology and rDNA sequence.

Isolation frequencies. —Ophiostoma ips, the only identified species, was isolated most frequently, from 85% of the 202 beetles sampled. Besides O. ips, the sterile fungus was isolated from 16% of the beetles, and the remaining species, including Sporothrix sp. C (from
3% of 202 beetles), Sporothrix sp. B (from 2.5%), and Hyalorhinocladiella sp. C (from 1.5%) were isolated infrequently (TABLE VII).

The chi-square test did not show differences in the frequency of fungal species isolated between the male vs. female beetles at the four different sites. However, there were differences in isolation frequencies of all isolated species, including O. ips, the sterile fungus, two Sporothrix spp. and Hyalorhinocladiella sp. among the four sites (Chi-squared is 40.2809, df = 12, and P < 0.0001). For instance, at the Porterville site, only O. ips was isolated. In contrast, at the Bakersfield, Fresno, and Visalia sites, four different species were isolated. The sterile fungus was found in Bakersfield with higher frequency (isolated from 34% of 76 beetles) than at the other sites (2% from Fresno beetles and 10% from Visalia beetles).

DISCUSSION

Among the five species of Ophiostomatales isolated from O. erosus in California, O. ips, the only identified species, was by far the most frequently isolated (from 85% of the 202 sampled O. erosus adults), and it had been reported as the most common associate for O. erosus in other countries (Ben Jamaa et al. 2007, Romón et al. 2007, Zhou et al. 2001). The sterile fungus was isolated with 16% of the adults, and the remaining species were isolated from less than 3% of the adults and may not be significant associates of O. erosus. Four of the five species were also associated with H. ligniperda in California (Chapter 2). The fungal species did not differ in incidence from male vs. female beetles, but the mycoflora among the four sites differed. Only one species, O. ips was isolated at the Porterville site, whereas three
more species, beside *O. ips*, were isolated at the other three sites. The sterile fungus was found in Bakersfield at a higher frequency (isolated from 34.2% of 76 beetles) than at the Fresno and Visalia sites.

*Ophiostoma ips* was first described from *Ips calligraphus* (Germar) on *Pinus echinata*, *P. sylverstris*, and *P. rigida* in eastern USA (Rumbold 1931). This species is an important and common fungus associated with various conifer-infesting bark beetles throughout the world (Ben Jamaa et al. 2007, Kirisits 2004, Mathiesen-Käärik 1960, Min et al. 2009, Reay et al. 2006, Romón et al. 2007, Rumbold 1931, Upadhyay 1981, Zhou et al. 2001, 2004, 2007). *Ophiostoma ips* is especially well known species in North America and Europe (Ben Jamaa et al. 2007, Kirisits 2004, Romón et al. 2007), and it has been introduced into Southern Hemisphere countries through the accidental introduction of various conifer-infesting bark beetles (Zhou et al. 2001, 2004, 2007). For example, *O. ips* was isolated from *O. erosus*, *Hylastes angustatus* (Herbst), and *H. ligniperda* (Fabricius) in South Africa (Zhou et al. 2001) and from *H. ater* (Paykull) and *H. ligniperda* at New Zealand (Reay et al. 2005, 2006).

The sterile fungus was isolated in relatively high frequency, and the same fungus was isolated from *H. ligniperda* in California at a low frequency (Chapter 2). This species was placed near *O. rectangulosporium* based on rDNA sequence comparison. *Ophiostoma rectangulosporium* does not produce conidia in culture (Ohtaka et al. 2002, 2006), and this species was not tied to a specific bark beetles in Japan (Ohataka et al. 2006, Yamaoka et al. 2004). Another *O. rectangulosporium*-like species was recently reported from pine-infesting bark beetles in China (Min et al. 2009) and Spain (Romón et al. 2007), but rDNA sequences
of isolates from China and Spain were not similar to the original *O. rectangulosporium* from Japan.

The ITS rDNA sequences of *Sporothrix* sp. B matched exactly with the sequences of *O. abietinum* (DQ396788) from *O. erosus* in South Africa (Zhou et al. 2006) and a *Sporothrix* species (AY280487 and AY280488) from Austria and Azerbaijan (Aghayeva et al. 2004). However, these isolates differ from the original description and isolate from the holotype of *O. abietinum* (CBS125.89 = AF484453) in size of conidia (4–7.5 × 1–2 µm) (Marmolejo and Butin 1990). The conidia of *Sporothrix* sp. B were similar to those of the *Sporothrix* species isolated from *P. echinata* in the USA (Aghayeva et al. 2004). The identification of *O. abietinum* in South Africa may have been in error.

*Sporothrix* and *Hyalohinocladiella* spp. are well known anamorphs of *Ophiostoma*, and unidentified species of these genera were infrequently isolated from *O. erosus* in California and elsewhere (Min et al. 2009, Reay et al. 2002, 2006, Romón et al. 2007, Yamaoka et al. 2004, Zhou et al. 2001, 2004, 2007). *Sporothrix* sp. C was isolated from *H. ligniperda* in California, but *Hyalohinocladiella* sp. C from *O. erosus* was different than the *Hyalohinocladiella* spp. isolated from *H. ligniperda* (chapter 2).

*Ophiostoma floccosum* Math.-Käärik, *O. olivaceum* Mathiesen, *O. piceae* (Münch) Syd. & P. Syd., *O. pluriannulatum*, *O. stenoceras*, *L. guttulatum*, *L. lundbergii*, *L. serpens*, and *L. wingfieldii* were associated with *O. erosus* in other countries (Romón et al. 2007, Zhou et al. 2001, 2006) but were not found in California. However, the frequency of occurrences of these species in the other countries was very low (less than 2% of the sampled beetles), except for *O. stenoceras* from Spain (Romón et al. 2007). *Ophiostoma stenoceras* is
closely related to *O. nigrocarpum* and *S. schenckii* (de Beer et al. 2003), and although these species were distinct from each other in rDNA sequence, they are often confused (Aghayeva et al. 2004, de Beer et al. 2003). Thus, isolates of *O. stenoceras* from Spain may have been misidentified and may be the same as one of the *Sporothrix* species isolated from *O. erosus* in California.

The results of this study have shown that *Ophiostoma* species are associated with *O. erosus* in California, and *O. ips* is a particularly common. This is consistent with the common association of *O. ips* with *Orthotomicus* in other countries. It is possible that the other species of Ophiostomatales isolated in California are not regular associates of this bark beetle.

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**LITERATURE CITED**


TABLE V. Species of *Ophiostoma* and related anamorph genera associated with *Orthotomicus erosus* in earlier studies.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Country</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ophiostoma abietinum</em></td>
<td>South Africa</td>
<td>Zhou et al. 2006</td>
</tr>
<tr>
<td><em>Ophiostoma floccosum</em></td>
<td>Spain</td>
<td>Romón et al. 2007</td>
</tr>
<tr>
<td></td>
<td>South Africa</td>
<td>Zhou et al. 2006</td>
</tr>
<tr>
<td><em>Ophiostoma ips</em></td>
<td>South Africa</td>
<td>Zhou et al. 2001</td>
</tr>
<tr>
<td></td>
<td>Spain</td>
<td>Romón et al. 2007</td>
</tr>
<tr>
<td></td>
<td>Tunisia</td>
<td>Ben Jamaa et al. 2007</td>
</tr>
<tr>
<td><em>Ophiostoma olivaceum</em></td>
<td>Spain</td>
<td>Romón et al. 2007</td>
</tr>
<tr>
<td><em>Ophiostoma piceae</em></td>
<td>Spain</td>
<td>Romón et al. 2007</td>
</tr>
<tr>
<td><em>Ophiostoma piliferum</em></td>
<td>South Africa</td>
<td>Zhou et al. 2006</td>
</tr>
<tr>
<td><em>Ophiostoma pluriannulatum</em></td>
<td>South Africa</td>
<td>Zhou et al. 2001</td>
</tr>
<tr>
<td></td>
<td>Spain</td>
<td>Romón et al. 2007</td>
</tr>
<tr>
<td><em>Ophiostoma stenoceras</em></td>
<td>Spain</td>
<td>Romón et al. 2007</td>
</tr>
<tr>
<td><em>Leptographium guttulatum</em></td>
<td>Spain</td>
<td>Romón et al. 2007</td>
</tr>
<tr>
<td><em>Leptographium lundbergii</em></td>
<td>South Africa</td>
<td>Zhou et al. 2001</td>
</tr>
<tr>
<td><em>Leptographium serpens</em></td>
<td>South Africa</td>
<td>Zhou et al. 2001</td>
</tr>
<tr>
<td><em>Leptographium wingfieldii</em></td>
<td>Spain</td>
<td>Romón et al. 2007</td>
</tr>
<tr>
<td><em>Pesotum fragrans</em></td>
<td>Spain</td>
<td>Romón et al. 2007</td>
</tr>
<tr>
<td><em>Pesotum spp.</em></td>
<td>South Africa</td>
<td>Zhou et al. 2001</td>
</tr>
<tr>
<td><em>Hyalorhinocladiaella</em> sp.</td>
<td>South Africa</td>
<td>Zhou et al. 2001</td>
</tr>
<tr>
<td><em>Sporothrix</em> sp.</td>
<td>South Africa</td>
<td>Zhou et al. 2001</td>
</tr>
</tbody>
</table>
TABLE VI. Location, hosts, and dates collected for male and female *Orthotomicus erosus* adults excavated from infested trees in California.

<table>
<thead>
<tr>
<th>County</th>
<th>City</th>
<th>Host species</th>
<th>Collection date</th>
<th>Number</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Kern County</td>
<td>Bakersfield</td>
<td><em>Pinus sp.</em></td>
<td>Mar 2006</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Tulare County</td>
<td>Porterville</td>
<td><em>Pinus sp.</em></td>
<td>Mar 2006</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Fresno County</td>
<td>Fresno</td>
<td><em>P. pinea, P. halepensis,</em></td>
<td>Dec 2005</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Kern County</td>
<td>Bakersfield</td>
<td><em>Pinus pinea</em> and <em>P. halepensis</em></td>
<td>Dec 2005</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Tulare County</td>
<td>Visalia</td>
<td><em>P. pinea</em> and <em>P. halepensis</em></td>
<td>Dec 2005</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>
TABLE VII. Number of successful isolations and isolation frequency (percent, in parentheses) of species in the Ophiostomatales isolated from adults of Orthotomicus erosus at four locations in California.

<table>
<thead>
<tr>
<th>Fungal Species</th>
<th>Bakersfield (n = 76)</th>
<th>Fresno (n = 50)</th>
<th>Porterville (n = 26)</th>
<th>Visalia (n = 50)</th>
<th>GenBank Accession No.</th>
<th>ITS</th>
<th>LSU</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ophiostoma ips</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterile fungus</td>
<td>27 (36%)</td>
<td>1 (2%)</td>
<td>0</td>
<td>4 (8%)</td>
<td>GU393357 GU393356</td>
<td>GU393353</td>
<td></td>
</tr>
<tr>
<td>(near <em>O. rectangulosporium</em>)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sporothrix</em> sp. C</td>
<td>1 (1.3%)</td>
<td>4 (8%)</td>
<td>0</td>
<td>0</td>
<td>GU393350 GU393349</td>
<td>GU393351</td>
<td></td>
</tr>
<tr>
<td>(near <em>S. schenckii</em>)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hyalorhinocladiella</em> sp. C</td>
<td>1 (1.3%)</td>
<td>0</td>
<td>0</td>
<td>3 (6%)</td>
<td>GU393355 GU393354</td>
<td>GU393354</td>
<td></td>
</tr>
<tr>
<td>No fungus isolated</td>
<td>5 (7%)</td>
<td>7 (14%)</td>
<td>0</td>
<td>1 (2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 GenBank accession numbers correspond to deposited sequences of representative isolates of each species.
TABLE VIII. Number and frequency of isolations of *Ophiostoma* and related species from adult *Orthotomicus erosus* collected from California (this study), and Spain and South Africa (earlier studies).

<table>
<thead>
<tr>
<th>Species</th>
<th>Countries</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spain <em>¹</em> (n = 219)</td>
<td>South Africa <em>²</em> (n = 665)</td>
<td>USA (n = 202)</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma canum</em>-like</td>
<td>2 (1%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma floccosum</em></td>
<td>2 (1%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma ips</em></td>
<td>43 (20%)</td>
<td>399 (60%)</td>
<td>172 (85%)</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma olivaceum</em></td>
<td>1 (0.4%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma piceae</em></td>
<td>4 (2%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma pluriannulatum</em></td>
<td>11 (5%)</td>
<td>2 (0.3%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma rectangulosporium</em>-like</td>
<td>3 (1.3%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Ophiostoma stenoceras</em></td>
<td>46 (21%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Leptographium guttulatum</em></td>
<td>11 (5%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Leptographium lundbergii</em></td>
<td>0</td>
<td>4 (0.6%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Leptographium serpens</em></td>
<td>0</td>
<td>3 (0.5%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Leptographium wingfieldii</em></td>
<td>1 (0.4%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Sterile fungus</td>
<td>0</td>
<td>0</td>
<td>32 (16%)</td>
<td></td>
</tr>
<tr>
<td><em>Hyalorhinocladiella</em> sp.*</td>
<td>0</td>
<td>1 (0.2%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Hyalorhinocladiella</em> sp. C</td>
<td>0</td>
<td>0</td>
<td>4 (2%)</td>
<td></td>
</tr>
<tr>
<td><em>Pesotum</em> spp.*</td>
<td>1 (0.4%)</td>
<td>11 (2%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><em>Sporothrix</em> spp.*</td>
<td>0</td>
<td>2 (0.3%)</td>
<td>11 (5.4%)</td>
<td></td>
</tr>
<tr>
<td>No fungus isolated</td>
<td>Not known</td>
<td>Not known</td>
<td>13 (6.4%)</td>
<td></td>
</tr>
</tbody>
</table>

¹ Romón et al. 2007
² Zhou et al. 2001
CHAPTER 4. GENERAL CONCLUSION

Two root-feeding, European bark beetles, *Hylurgus ligniperda* and *Orthotomicus erosus* (Coleoptera: Curculionidae: Scolytinae), were recently introduced to Los Angeles, California in 2003 and southern California in 2004, respectively. These bark beetle species are common vectors of Ophiostomatales, some of which cause blue-stain of conifer sapwood. Most blue-stain fungi associated with conifer bark beetles belong to the genera *Ceratocystiopsis*, *Ophiostoma*, *Grosmannia*, and related anamorphic genera such as *Leptographium*, *Pesotum*, *Hyalorhinocladiella*, and *Sporothrix*. These Ophiostomatales produce sticky spore at the tip of perithecia or wet droplets of conidia, and the spore masses may be adhere to the exoskeleton of bark beetles and may be transmitted tree-to-tree.

In this study, 118 adult *H. ligniperda* and 202 adult *O. erosus* were collected from infested pines, in California. From these specimens, 114 individual isolates of Ophiostomatales were obtained from *H. ligniperda* and 219 from *O. erosus* on cycloheximide-amended media. Overall, 15 species of Ophiostomatales (eight identified species and seven unidentified species) were isolated from *H. ligniperda* and five Ophiostomatales (one identified species and four unidentified species) were isolated from *O. erosus*.

The frequency of occurrence of species from *H. ligniperda* were as follows: *O. ips* (isolated from 31% of the 118 beetles), *G. galeiforme* (23%), *O. piceae* (9%), *O. querci* (8%), *L. tereforme* sp. nov. (6%), *G. huntii* (4%), *L. serpens* (3%), three different *Sporothrix* spp.
(7%), *O. floccosum* (2%), *O. stenoceras* (1%), a sterile fungus (1%), and two different *Hyalorhinocladiella* spp. (2%). In contrast, *O. ips* (isolated from 85% of the 202 beetles), a sterile fungus (16%), two different *Sporothrix* spp. (5%), and a *Hyalorhinocladiella* sp. (1%) were isolated from *O. erosus*.

*Grosmannia galeiforme*, *L. tereforme* and a sterile fungus were recorded from the USA for the first time, and *L. tereforme* is described as new. *Leptographium tereforme* was morphologically most similar to *G. robustum* because of its globose conidia and granular material on the conidiophores and hyphae, but it can be distinguished by rDNA sequences. On the other hand, the sterile fungus did not produce a teleomorph or conidial stage in culture, but its rDNA sequences were similar to those of *O. rectangulosporium*.

*Ophiostoma ips*, the sterile fungus, *Sporothrix* spp., and *Hyalorhinocladiella* spp. were isolated from both *H. ligniperda* and *O. erosus*, and *O. ips* was the most frequently encountered species from both *H. ligniperda* and *O. erosus*.

The strains of *O. ips* associated with these beetles may have been brought to California because they had been reported as common associates of these beetles in other countries. *Grosmannia galeiforme*, *L. serpens*, and perhaps the sterile fungus were likely introduced to California with the bark beetles. Otherwise, the isolated Ophiostomatales from both *H. ligniperda* and *O. erosus* in California were isolated infrequently and most were well known in the USA and throughout the world.
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I am grateful to my Lord God that he has been with me during my studies and led me to begin studying in plant pathology and opened a way to complete this thesis when I had no ability to do it. I am grateful to my husband, Minhui, and my daughter, Annette. Thank you for being a good husband, best friend and great father, also thank you for all your love, supports and prayers. My deepest gratitude goes to my parents for their unlimited love and ceaseless support throughout my life.

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