Leptographium tereforme sp. nov. and other Ophiostomatales isolated from the root-feeding bark beetle Hylurgus ligniperda in California

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INTRODUCTION

Hylurgus ligniperda (Fabricius) (Coleoptera: Scolytidae, sensu Bright 1993, Wood 2007), 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 was introduced accidentally to Chile, New Zealand and South Africa, and it recently was introduced into the USA. The first established breeding population in North America was discovered in 2000 in New York, although specimens had been collected from the same general area as early as 1994. Hylurgus ligniperda also was detected in July 2003 in Los Angeles, California, and is abundant and well established from northern Los Angeles and Ventura counties to San Diego County in southern California (Liu et al. 2007).

Hylurgus ligniperda is a root- and stump-infesting beetle whose elytral declivity is covered with reddish hairs, hence its common name, the redhaired pine bark beetle (Lee et al. 2007). 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 to 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, Ceratocystis, Grosmannia and Ceratocystis. These genera represent two phylogenetically unrelated groups, Ophiostomatales, close to Diaporthales, and Ceratocystis, in Microascales (Harrington 2005, Harrington et al. 2010, Zipfel et al. 2006). Most blue-stain fungi associated with conifer bark beetles belong in Ophiostomatales, although a few conifer bark beetles transmit Ceratocystis species (Harrington 2005, Kiritis 2004). Sexual species in 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). Zipfel et al. (2006) redefined Ophiostoma and distinguished Ophiostoma species...
(with *Pesotum*, *Hyalorhinocladiella* and *Sporothrix* anamorphs) from *Ceratocystis* (with *Hyalorhino-
cadiella* anamorphs) and *Grosmannia* (with *Leptogra-
phium* anamorphs). Mullineux and Hausner (2009) studied the secondary structure of the internal transcribed spacer regions of rDNA and further supported separation of *Grosmannia* from *Ophiostoma*, but there is still some question as to the monophyly of *Grosmannia* (Harrington et al. 2010).

Ophiostomatales have been isolated from *H. ligniperda* collected in South Africa (Zhou et al. 2001), Chile (Zhou et al. 2004a), New Zealand (Reay et al. 2006) and Spain (Romón et al. 2007). Zhou et al. (2001) reported eight Ophiostomatales from *H. ligniperda* that were obtained from infested stumps and root collars of *Pinus patula* and *P. elliottii* in South Africa, *Ophiostoma ips*, *L. lundbergii* Lagerb. & Melin and *L. serpens* were commonly isolated species, and *Ceratocystis minuta* (Siemaszko) Upadh. & Kendrick, *G. galeiforme*, *O. piceae*, *O. stenoceras* and *O. floccosum* in % adults in Spain. The *H. u* and 83 anamorphs) and *Hyalorhino-

2 (C1018, mat-a, and *L. lundbergii*) *G. huntii* were isolated


Ophios-

were collected from *O. piceae* anamorphs). Mullineux and Hausner (2009) collected in South Africa (Zhou et al.

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In Mar 2006 118 adult *O. ips* were collected 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 1 L 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. 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.

Ten day old cultures were incubated at 25 C on MEA. Thirty measurements were made for each character from each isolate studied, and the mean and range were computed for each character from each isolate. Colonized agar plugs (5 mm diam) were taken from 10 d cultures and placed in the middle of fresh MEA plates, mycelium side down, for a temperature growth study. 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), *G. galeiforme* (DM269-1, mat-a, and DM269-2, mat-b, from Scotland), *G. 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 twig) (Harrington 1992). Debarked twigs of *Pinus strobus* L. were cut 5–4 cm long and autoclaved twice for 30 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 PTM until fully colonized and the mycelium was exposed to spermatozoa with 20 µL conidial slurry of the second isolate (Harrington and McNew 1997).

DNA sequencing and RFLP analysis.—Each isolate was grown at room temperature 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 with 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 with primers LROR and LR5 (Vilgalys and Hester 1990). The ITS regions and the 5.8S gene of the ribosomal RNA operon were amplified with primers ITS1-F (Gardes and Bruns 1993) and ITS-4 (White et al. 1990). Template DNA was amplified in a 50 µL single reaction volume, containing 1.25 units Takara Ex Taq Polymerase, 1× PCR reaction buffer, 200 µM dNTPs, 5% (V/V) DMSO, and 0.25 µM of each primer (Takara Mirus Bio, Japan). Cycling conditions were 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 2 min, followed by one final cycle of primer extension at 72 °C 15 min (Harrington et al. 2001).

PCR products either were purified with a QIAquick PCR Purification Kit (QIAGEN Inc., California) or were digested with restriction enzyme. PCR products were sequenced at the DNA Synthesis and Sequencing Facility at Iowa State University with primers ITS1-F and ITS-4 for the ITS region or LROR and LR3 (Vilgalys and Hester 1990) for the LSU.

The restriction enzyme HaeII (Gibco BRL Inc., USA), which recognizes the base sequence RGGC/G/Y, was used to identify members of the O. piceae complex (Harrington et al. 2001). Each unpurified PCR product (10 µL) was mixed with 2 µL 10× buffer (supplied with the enzyme), 1 µL restriction enzyme, and 7 µL sterilized, distilled water. Digestion was allowed to proceed 3 h at 37 °C, and then restriction fragments were separated by electrophoresis 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 with MegaBLAST (BLASTN 2.2.22 +).

RESULTS

Isolation of fungi from Hylurgus ligniperda.—In total 118 H. ligniperda adults were sampled for the presence of Ophiostomatales and 114 individual Ophiostomatales isolates were obtained on CSMA. Of the 118 beetles 87 (74%) yielded at least one species of Ophiostomatales. Twenty-two percent of 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 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 Hyalorhinocadiella anamorphs. Another species produced no spores in culture.

Many species were similar to common Ophiostoma and Leptographium species (pigmented and macro-nematous conidiophores) reported from H. ligniperda from the southern hemisphere. The most common Ophiostoma species were distinguished by comparing rDNA sequences and morphological descriptions of Ophiostomatales. Overall eight species were identified but seven 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. O. ips was easily distinguished morphologically from other fungi by brown 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 diam, with straight or curved necks, 180–1100(–1300) µm long, and no ostiolar hyphae. Ascospores oblong, surrounded by a hyaline gelatinous sheath, appearing rectangular or square, 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. 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 galleiforme (Bakshi) Zipfel Z.W. de Beer & M.J. Winf. All G. galleiforme isolates had light gray to dark brown mycelium and symnematous conidiophores (common) and rarely mononematous conidiophores. These isolates produced perithecia and ascospores on PTM 6–8 wk when paired with mating testers. Perithecia bases, (120–)195–240(–280) µm diam, necks (510–)600–710 µm long. Ascospores reniform with sheath, 1.8–4.3 × 1.0–2.4 µm. Synnematata 120–300 µm tall. Conidia cylindrical, (2–)3.2–5 × 1–2.5 µm. ITS sequences of all G. galleiforme isolates (e.g. GU129981) matched (514 of 514 nucleotides) with the previously reported sequences of G. galleiforme (AY649770, DQ062979).

Ophiostoma piceae (Mińch) 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 diam, and straight necks, 610–960 µm long. Ascospores reniform, (2–)2.5–4.5 ×
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. (AY935606), G. clavigerum (Rob.-Jeffr. & Davids.), Zipfel Z.W. de Beer & M.J. Winf. (AY263196), G. robustum (Rob.-Jeffr. & Davids.), Zipfel Z.W. de Beer & M.J. Winf. (AY263190, AW263189), L. longiclavatum (Lee, Kim & Breuil (AW11668), L. terebrantis (Barras & Perry (AY935607) and L. pyrimum Davidson (AY44621). 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, although 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-Jeffery and Grinchenko 1964), but Leptographium sp. A formed no perithecia in culture, even when isolates were paired. 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. Conidigenous 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. 2003a) 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 (EF127884, 527 of 536 base pairs matching and EF127882, 530 of 536 bases matching).

Grosmannia huntii (Robins.-Jeffr.) Zipfel Z.W. de Beer & M.J. Winf. Isolates of G. huntii failed to produce perithecia, even when paired with the mat-a tester of G. huntii from Chile. However the Leptographium state of the California isolates was consistent with reports for G. huntii (Jacobs et al. 1998, Jacobs and Wingfield 2001, Robinson-Jeffery and Grinchenko 1964). 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 ovoid with truncate bases and rounded apices, (3–)4.5–5.4 (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 (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) 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. L. serpens originally was described from Pinus sylvestris L. 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 instead of 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 O. serpens, but the sequence was shorter (Jacobs and Wingfield 2001).
to accessions AY554488 and AY707203 of *L. serpens* (81 bases matching). The LSU sequence (GU129998) of *L. serpens* matched closely 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. *O. floccosum* isolates from California did not form perithecia and ascospores when paired with testes of opposite mating type, but they did produce typical *Pestalotia* 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 HadII 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 diam 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 diam, 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, AF280492 and AF484476.

Sterile fungus. This species did not produce perithecia or conidia on MEA, MYEA or PTM. *Colonies* white and fast-growing, filling an 85 mm 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. rectangulosporum* 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. rectangulosporum* (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 the 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). ITS sequences of isolates of *Sporothrix* sp. B (GU129985) matched those of southern pine beetle isolates C349 and C558 of *O. nigrocarpum* (541 of 542 bases matching) and were similar to those of *O. abietinum* Marmolejo & Butin (DQ596788 and AF484453, 538 of 538 bases matching).

Hyalorhinocladiella sp. A. *Colonies* white, with no aerial mycelium. *Hyphae* thick-walled, submerged under the medium. *Conidiophores* arising directly from hyphae, lacking prominent denticles. *Conidia* broadly ellipsoidal to subcylindrical, 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. rectangulosporum* 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 (GU129998) 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. (AY456710, 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.

*MycoBank* MB518036

Colonieae olivacea vel atro-viridae, ad 34 mm in 10 dies in 1.5% MEA. *Hyphae* brunnea, granulatae. *Conidiophorae* singulæ aut aggregatae, (35–)70–150 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 conidia.


Colonies with optimal growth at 25 C on 1.5% MEA, attaining 35 mm diam in 10 d, olive green to dark green or dark gray with age. No growth at 5 C or 35 C. Hyphae mostly submerged, covered by granular material (Fig. 1D). Perithecia and ascospores absent. Conidiophores occurring singly or in groups (3–4), micronematous, mononematous, (35–)70(–130) μm long (Fig. 1A, 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 (Fig. 1C). Primary branches, if present, hyaline, smooth, cylindrical, (8–)19–18(–30) μm long. Secondary branches absent or present, hyaline, (5–)10(–15) μm in long. Conidia hyaline, aseptate, oblong to slightly obvoid with round apices, 3–5(–8) × 2.5–4.0(–5) μm (Fig. 1E).

Conidial droplet hyaline at first, becoming creamy yellow, remaining the same color when dry.

Isolation frequencies.—*Ophiostoma ips* was isolated from 31% of 118 beetles sampled. In addition to *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 each were isolated from fewer than 5% of beetles (Table I). Each of the other species, including *O. stenoceras*, were isolated only from one beetle.

No difference in frequency of isolation was observed between male vs. female beetles at either site for any of the fungal species based on a Chi-square analysis (*P = 0.05*). However there were differences in frequency of isolation of some species between the two sites. At San Dimas *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 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 the sterile fungus were found only at La Cañada Flintridge (Table I).

**DISCUSSION**

Fifteen Ophiostomatales species were isolated from the root-feeding bark beetle *Hylurgus ligniperda* in California. Most of the previously isolated species had been reported from USA, mostly as blue-stain fungi on pine sapwood (Jacobs and Wingfield 2001, Harrington 1988, Harrington et al. 2001, Upadhyay 1981). *Ophiostoma ips*, *O. piceae*, *O. querci*, *O. floccosum* and *O. stenoceras* are commonly isolated species from *H. ligniperda* and have been reported from other bark beetles in North America and worldwide (Harrington et al. 2001, Reay et al. 2006, Romón et al. 2007, Zhou et al. 2001, 2004a). Fungi associated with *H. ligniperda* on other continents but not found in western North America included *G. galeiforme* and *L. serpens*, so it is possible that these two species were introduced to California with the beetle.

Of all the species isolated in this study *G. galeiforme* was isolated consistently from *H. ligniperda* at both California sites. This species is believed to be European (Bakshi 1951, Mathiesen-Käärik 1960) and has been associated with a wide range of European
bark beetles, including *Orthotomicus erosus* (Wollaston), *Hylastes 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* was introduced into the southern hemisphere 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* had not been confirmed. Thus it is likely that *H. ligniperda* brought *G. galeiforme* with it when it was accidentally introduced to California. To date, pathogenicity of *G. galeiforme* to pine has not been reported, so the finding of this fungus in California might not be cause for concern.

*Leptographium serpens* has been isolated from European root-feeding bark beetles, including *H. angustatus* and *H. ligniperda* and *Hylastes* spp., in South Africa and elsewhere (Harrington 1988, Jacobs and Wingfield 2001, Reay et al. 2002, 2005, 2006; Thwaites et al. 2005; Wingfield et al. 1988; Zhou et al. 2001, 2002, 2004a). For example 45% of Ophiostomatales isolates from *H. angustatus* in South Africa were *L. serpens* and 21% of *H. ligniperda* isolates were *L. serpens* (Zhou et al. 2001). *Leptographium serpens* has been associated with roots of declining *Pinus* spp. in southeastern USA (Eckhardt et al. 2007). *Leptographium serpens* has not been reported from western North America, but it was isolated from *H. ligniperda* at both sites in California, although at low frequency. Because *L. serpens* had not been reported from western North America but it has been commonly associated with European bark beetle species such as *H. ligniperda* it is likely that this beetle brought the fungus with it to California. *Leptographium serpens* has not been considered a serious pathogen of *Pinus* spp. (Wingfield et al. 1988, Zhou et al. 2002) or as a contributor to loblolly pine decline (Eckhardt et al. 2007) in southeastern USA, and *L. serpens* was only weakly pathogenic to wounded loblolly pine seedlings under low soil moisture conditions (Matustick et al. 2008). However the presence of *L. serpens* in California needs to be monitored, and further pathogenicity tests might be warranted.

The new species, *Leptographium tereforme*, was isolated from 6% of *H. ligniperda* but only at La Cañada Flintridge; thus *H. ligniperda* might have acquired the fungus from another bark beetle. This fungus is distinguished from related species, such as *G. aureum*, *G. clavigerum*, *G. robustum*, *L. longiclavatum*, *L. terebrantis* and *L. pyrinum*, 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) Wingfield, *L. hundbergii* and *L. guttulatum* M.J. Wingf. & Jacobs. *Leptographium tereforme* is easily distinguished morphologically from these species by its globose conidia, short conidio- phores and granular material covering half or up to three-quarters of the conidioaphore stipe. *L. tereforme* is not known elsewhere, but it is possible that reports of *L. guttulatum*, *L. hundbergii* 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*.

*Ophiostoma ips* is known worldwide as a blue-stain 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, Romón et al. 2007, Rumbold 1931, Seifert 1993). The fungus was taken to 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 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 13% of 199 sampled *H. ligniperda* adults (Zhou et al. 2001). In contrast this species was reported with a high frequency of occurrence in isolations from *H. ligniperda* in Chile (Zhou et al. 2004a). At La Cañada Flintridge *O. ips* was isolated from only 11% of beetles, although *O. ips* was isolated from 62% of beetles sampled from San Dimas. The high frequency of occurrence of *O. ips* at the latter site might 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* the beetle might have acquired *O. ips* in California.

*Ophiostoma piceae* and *O. querci* were isolated frequently from *H. ligniperda* collected from La Cañada Flintridge, and *O. floccosum* was isolated from two beetles at this site. These synnema-forming *Ophiostoma* species were not isolated from beetles collected at San Dimas. 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* originally was 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* often were treated as synonyms of *O. piceae* (Harrington et al. 2001); thus former studies with *H. ligniperda* might have considered *O. querci* and *O. floccosum* isolates as *O. piceae*. The association of *O. piceae* and *O. querci* with *H. ligniperda* was reported from 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* (L.), but not from *H. ligniperda*. In the New Zealand study (Reay et al. 2006) synnema-forming species were distinguished and *O. querci*, *O. floccosum* and *O. setosum* were identified from *H. ligniperda*. However these species are not strict associates of *H. ligniperda* and often are encountered in the absence of bark beetle activity (Harrington et al. 2001; Reay et al. 2002, 2006; Thwaites et al. 2005).

*Grosmannia huntii* first was described from pine infested with a *Dendroctonus* sp. in Canada (Robinson-Jeffrey and Grinchenko 1964). *Grosmannia huntii* has been confused with *G. piceaperda* (Rumbold) Goid., but Jacobs et al. (1998) separated the species by morphology and mating system. *Grosmannia huntii* has been associated with many bark beetle species on *Pinus* and *Picea* spp.; beetle species include *D. ponderosae* Hopkins, *Ips pini* (Say), and *Hylastes macer* (LeConte) in the USA (Harrington 1988, Jacobs and Wingfield 2001), *Tomicus piniperda* (L.) in Europe (Gibbs and Inman 1991) and *H. ligniperda* in New Zealand, where *G. huntii* was isolated from 83% of *H. ligniperda* adults (Reay et al. 2006); 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.

Other species isolated from *H. ligniperda* in California were from only one or two beetles. *Ophiostoma stenoceras* has been isolated frequently 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 might 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 somewhat 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 *Cryptalus 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.

A number of species were isolated from *H. ligniperda* in other studies but were not identified among the California isolations. *Leptographium guttulatum* has been associated with bark beetles in the genera *Dryocoetes, Hylastes, Hylurgops* and *Tomicus* (Jacobs et al. 2001, Romón et al. 2007, Wingfield and Gibbs 1991). *Leptographium truncatum* was isolated rarely from *H. ligniperda* in New Zealand (Reay et al. 2006). *Leptographium lundbergii* was reported from South Africa (Zhou et al. 2001), but the taxonomy of this species and *L. truncatum* had been confused (Jacobs and Wingfield 2001, Jacobs et al. 2005, Strydom et al. 1997, Zambino and Harrington 1992) and it is possible that Zhou et al. (2001) might have misidentified *L. truncatum* or *L. tereforme* isolates as *L. lundbergii*. It also is possible that some fungi present on *H. ligniperda* in California were not isolated because the beetles were killed before shipping to Iowa. However this beetle is not known to have a mycangium, and spores of Ophiostomatales on the exoskeleton of the beetle probably would survive in high numbers at low temperatures for a few days. Isolation frequencies of *O. novo-ulmi* Brasier from killed *Scolytus scheff рейvi* Semenov that were shipped from Colorado to Iowa were just as high as from beetles that were plated immediately after killing (Jacobi et al. 2007).

Our results showed that a relatively large number of *Ophiostoma* species are associated with *H. ligniperda* in California. Most of the species isolated are common bark beetle associates in USA and around the world, and many of the species have been associated with *H. ligniperda* in the southern hemisphere. It is likely 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 through exchange of Ophiostomatales associated with cohabiting native bark beetles.

*H. ligniperda* recently was detected in northern Los Angeles County in native stands of single-leaf pinyon
pine, *Pinus monophylla* Torr. & Frem. (D-G Liu pers comm), which raises the question whether *H. ligniperda* could become associated with and become a vector for *L. wageneri* (Kendr.) Wingfield, the cause of black-stain root disease of conifers (Harrington 1988, 1993). In this context it will be important to examine the feeding and host colonization of *H. ligniperda* on healthy pine roots and to examine populations of *H. ligniperda* collected in stands of *P. monophylla* for their associated fungi.

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