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

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Abstract: The redhaired pine bark beetle *Hylurgus ligniperda* (F.) is native to Europe but was discovered in Los Angeles, California, in 2003. This root- and stump-feeding 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 logs of *Pinus halepensis* and *P. pinea* at two sites in California. In total eight species of Ophiostomatales were identified and seven species that occasionally were isolated were unidentified. The most frequently isolated species were *Ophiostoma ips* and *Grosmannia galeiforme*, which were isolated respectively from 31% and 23% of the 118 beetles. The other species isolated included *O. piceae* (isolated from 9% of the beetles), *O. querci* (8%) and *Leptographium tereforme* sp. nov. (6%). *Grosmannia huntii, L. serpens*, three *Sporothrix* species, *O. floccosum, O. stenoceras*, two unidentified *Hyalorhinocladiella* sp. and a sterile fungus each were isolated from fewer then 5% of beetles. Most of the identified species already were known in 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 USA for the first time, and this is the first report of *L. serpens* from western North America.

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

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, Ceratocystiopsis, 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, Kirisits 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 *Ceratocystiopsis* (with *Hyalorhinocladiella* anamorphs) and *Grosmannia* (with *Leptographium* 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 *Ceratocystiopsis minuta* (Siemaszko) Upadh. & Kendrick, *G. galeiforme, O. piceae, O. stenoceras* and *O. pluriannulatum* (Hedgc.) H. & P. Sydow were isolated from fewer than 2% of beetles (Zhou et al. 2001). Reay et al. (2006) isolated respectively *G. galeiforme* and *G. huntii* from 96% and 83% of adult *H. ligniperda* in New Zealand; *O. floccosum, O. setosum* Uzunovic, Seifert, S.H. Kim & C. Breuil, *O. ips, O. querci, L. procerum* (W.B. Kendr.) M.J. Wingf. and *L. truncatum* (M.J. Wingf. & Marasas) M.J. Wingf. were isolated less frequently. Zhou et al. (2004a) isolated only *C. minuta, G. galeiforme* and *O. ips* from 80 *H. ligniperda* adults collected in Chile. Romón et al. (2007) isolated only *L. guttulatum* M.J. Wingf. & K. Jacobs from five *H. ligniperda* adults in Spain. The fungi associated with *H. ligniperda* in USA have not been documented.

This study explored the relationship between *H. ligniperda* and Ophiostomatales in California to examine whether the beetle brought potential tree pathogens or new blue-stain fungi to North America and whether the beetle might have begun carrying native North American fungi.

MATERIALS AND METHODS

Beetle collection and isolation.—In Mar 2006 118 adult *H. ligniperda*, 55 females and 63 males, were collected live from egg galleries in the phloem (inner bark) of cut stem sections of pine at two sites in California. Seventy-three adult *H. ligniperda* were collected from *Pinus halepensis* Mill. and *P. pinea* L. at Descanso Gardens (La Cañada Flintridge, 34.21055°N, 118.20044°W), and 45 adult *H. ligniperda* were collected from *P. halepensis* and *P. pinea* at Bonelli Regional Park (San Dimas, 34.08856°N, 117.81253°W). Adults were placed individually 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 flame-sterilized forceps and shipped from California to Iowa, where the beetles were refrigerated until processing.

Each beetle was cut aseptically into four parts (head, prothorax, mesothorax and abdomen), and each part was placed in cycloheximide-streptomycin malt agar (CSMA, 10 g malt extract, 15 g agar, 200 mg cycloheximide and 100 mg streptomycin per 1 L dH$_2$O) (Harrington 1981, 1992). 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 1 L dH$_2$O). 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 3–4 cm long 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 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 dH$_2$O), 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 *Hae*II (Gibco BRL. Inc., USA), which recognizes the base sequence RGCGC/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 procede 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 con-

tained *Leptographium* anamorphs, the third group contained only *Sporothrix* anamorphs and the fourth group had *Hyalorhinocladiella* anamorphs. Another species produced no spores in culture.

Many species were similar to common *Ophiostoma* and *Leptographium* species (pigmented and macronematous 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, AYl72021 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 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. *Synnemata* 120–300 μm tall. *Conidia* cylindrical, (2–)3.2–5 × 1–2.5 μm. ITS sequences of all *G. galeiforme* isolates (e.g. GU129981) matched (514 of 514 nucleotides) with the previously reported sequences of *G. galeiforme* (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 diam, 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 *O. piceae* isolates had the same *Hae*II restriction pattern, with fragments of 400, 200, 120 bp, as had been reported 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* (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). All *O. querci* isolates had *Pesotum* and *Sporothrix* synanamorphs. *Perithecia* with globose bases, 150–215 µm diam, 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 *O. querci* isolates had the same *Hae*II restriction pattern, with fragments of 520 and 200 bp, consistent with 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. (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, 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, 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-Jeffrey 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. *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. 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* (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 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-Jeffrey 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* 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 (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 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 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 *Hae*II 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, 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 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. 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. nirgrocarpum [*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 541 bases matching) and were similar to those of *O. abietinum* Marmolejo & Butin (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 hyphae, lacking prominent denticles. *Conidia* broadly ellipsoidal to subcylindrical, 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. *Condiophores* 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.   Fig. 1

MycoBank MB518036

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
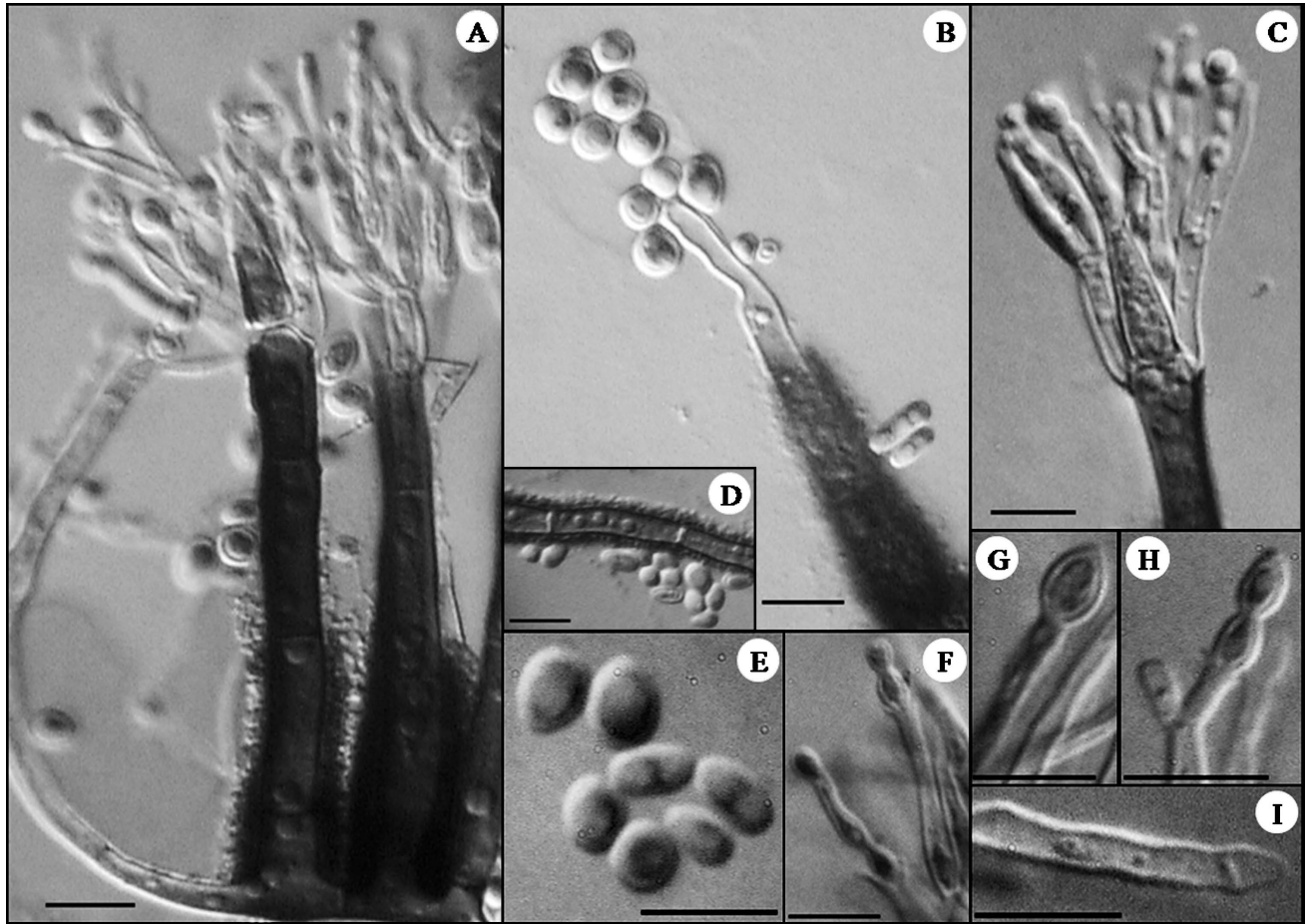
FIG. 1. Condiophores, conidia and hyphae of *Leptographium tereforme*. A–C. Conidiophores with stipes covered with brown, granular material. A, C. Conidiophores with primary and secondary metulae. B. Conidiophore with no metulae. D. Hyphae covered with granular material. E. Conidia. F–I. Conidiogenous cells showing holoblastic and enteroblastic conidium formation. Bars: A–C = 20 µm, D–I = 10 µm.

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.

Additional cultures examined. USA. CALIFORNIA: Los Angeles County, La Cañada Flintridge, Descanso Gardens, from female *H. ligniperda*, 26 Mar 2006, *S.J. Kim, C2409*; Los Angeles County, La Cañada Flintridge, Descanso Gardens, from male *H. ligniperda*, 26 Mar 2006, *S.J. Kim, C2421*; Los Angeles County, La Cañada Flintridge, Descanso Gardens, from male *H. ligniperda*, 26 Mar 2006, *S.J. Kim, C2422*; Los Angeles County, La Cañada Flintridge, Descanso Gardens, from female *H. ligniperda*, 26 Mar 2006, *S.J. Kim, C2426.*

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–)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. 1E).

TABLE I. Number of successful isolations and frequency (percent, in parentheses) of *Ophiostoma* and related species from adult *Hylurgus ligniperda* from New Zealand, La Cañada Flintridge, and San Dimas, California and representative ITS and LSU rDNA sequences

Species	New Zealand[1] (n = 120)[2]	California, USA		GenBank accession number[3]	
		La Cañada Flintridge (n = 73)	San Dimas (n = 45)	ITS	LSU
Grosmannia galeiforme	115 (95.8%)	18 (24.6%)	9 (20%)	GU129981	GU221908
Ophiostoma ips	6 (5%)	8 (11%)	28 (62%)	GU129980	
Ophiostoma piceae	0	11 (15%)	0	GU129988	
Ophiostoma querci	2 (1.7%)	10 (13.7%)	0	GU129989	
Leptographium tereforme	0	7 (9.6%)	0	GU129994	
Grosmannia huntii	100 (83.3%)	2 (2.7%)	3 (6.7%)	GU129982	GU129999
Sporothrix sp. C	0	4 (5.5%)	1 (2.2%)	GU129986	
Leptographium serpens	0	2 (2.7%)	2 (4.4%)	GU129983	GU129998
Ophiostoma floccosum	6 (5%)	2 (2.7%)	0		
Sporothrix sp. A	0	1 (1.4%)	1 (2.2%)	GU129984	GU221907
Ophiostoma stenoceras	0	1 (1.4%)	0	GU129990	
Sporothrix sp. B	0	1 (1.4%)	0	GU129985	
Hyalorhinocladiella sp. A	0	1 (1.4%)	0	GU129997	GU221905
Hyalorhinocladiella sp. B	0	1 (1.4%)	0	GU129996	GU221906
Sterile fungus	0	1 (1.4%)	0	GU129987	
Ophiostoma setosum	7 (5.8%)	0	0		
Leptographium procerum	7 (5.8%)	0	0		
Leptographium truncatum	1 (0.8%)	0	0		
No fungus isolated	Not available	24 (32.9%)	8 (17.8%)		

[1] Data from Reay et al. 2006.
[2] Number of adult beetles sampled.
[3] GenBank accession numbers correspond to sequences of representative isolates of each species.

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. long-*

iclavatum, *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. lundbergii* and *L. guttulatum* M.J. Wingf. & Jacobs. *Leptographium tereforme* is easily distinguished morphologically from these species by its globose conidia, short conidiophores and granular material covering half or up to three-quarters of the conidiophore stipe. *L. tereforme* is not known elsewhere, but 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*.

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. piceaperdum* (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 *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.

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 schevyrewi* 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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