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Identification, pathogenicity and abundance of *Paracremonium pembeum* sp. nov. and *Graphium euwallaceae* sp. nov.—two newly discovered mycangial associates of the polyphagous shot hole borer (*Euwallacea* sp.) in California

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Abstract: *Fusarium euwallaceae* is a well-characterized fungal symbiont of the exotic ambrosia beetle *Euwallacea* sp. (polyphagous shot hole borer [PSHB]), together inciting Fusarium dieback on many host plants in Israel and California. Recent discoveries of additional fungal symbionts within ambrosia beetle mycangia suggest these fungi occur as communities. Colony-forming units of *Graphium euwallaceae* sp. nov. and *Paracremonium pembeum* sp. nov., two novel fungal associates of PSHB from California, grew from 36 macerated female heads and 36 gallery walls collected from *Platanus racemosa, Acer negundo, Persea americana* and *Ricinus communis*. Fungi were identified based on micromorphology and phylogenetic analyses of the combined internal transcribed spacer region (nuc rDNA ITS1- 5.8S-ITS2 [ITS barcode]), elongation factor (*EF 1-α*), small subunit (18S rDNA) sequences for *Graphium* spp., ITS, *EF 1-α*, calmodulin (*cmdA*), large subunit of the ATP citrate lyase (*acl1*), β-tubulin (*tub2*), RNA polymerase II second largest subunit (*rpb2*) and large subunit (28S rDNA) sequences for *Paracremonium* spp. Other *Graphium* spp. recovered from PSHB in Vietnam, *Euwallacea fornicatus* in Thailand, *E. validus* in Pennsylvania and *Paracremonium* sp. recovered from PSHB in Vietnam were identified. *F. euwallaceae* was recovered from mycangia at higher frequencies and abundances in all hosts except *R. communis*, in which those of *F. euwallaceae* and *P. pembeum* were equal. *P. pembeum* was relatively more abundant within gallery walls of *A. negundo* and *R. communis*. In all hosts combined *F. euwallaceae* was relatively more abundant within PSHB heads than gallery walls. All three fungi grew at different rates and colonized inoculated excised stems of *P. americana* and *A. negundo*. *P. pembeum* produced longer lesions than *F. euwallaceae* and *G. euwallaceae* on inoculated avocado shoots. Results indicate PSHB is associated with a dynamic assemblage of mycangial fungal associates that pose additional risk to native and nonnative hosts in California.

Key words: ambrosia fungi, avocado, fungal associates, fungal pathogens, mycangia, symbiosis

Introduction

Fusarium dieback is a recently discovered beetle-associated disease complex that emerged in southern California in early 2012 (Eskalen et al. 2012). While most of the recent invasive ambrosia beetle/fungus complexes do not cause harm in their native range (Hulcr and Dunn 2011), this beetle/disease complex appears capable of infesting living trees both in its

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invaded and native range. The cause of the dieback is the combined action of the mechanical beetle burrowing and localized pathogenicity of the newly described fungal species *Fusarium euwallaceae* S. Freeman, Z. Mendel, T. Aoki et O'Donnell, a symbiotic fungus of the polyphagous shot hole borer (PSHB, *Euwallacea* sp. Coleoptera: Scolytinae), an invasive ambrosia beetle first reported in California in 2003 (Mendel et al. 2012, Eskalen et al. 2013, Kasson et al. 2013). Together these agents cause cambial necrosis, branch dieback and tree mortality on avocado and other hosts in Israel (Mendel et al. 2012), colonizing more than 139 host species in California, many of them as outwardly healthy trees, of which 38 are suitable for reproduction by the beetle (Eskalen et al. 2013). The host range continues to grow and is frequently updated (http://eskalenlab.ucr.edu/avocado.html). Multilocus phylogenetic analysis revealed that *F. euwallaceae* isolates from Israel and California were uniform phylogenetically (Eskalen et al. 2012, Freeman et al. 2013) but distinct from *F. ambrosium* and other *Euwallacea*-associated fusaria from Australia, Florida, India, Malaysia, Pennsylvania, San Diego, California, Singapore and Sri Lanka (Kasson et al. 2013, O'Donnell et al. 2015). The exotic beetle from the Los Angeles Basin in California (referred to herein as LA, which includes Los Angeles, Orange, Riverside and San Bernardino counties) and Israel is morphologically indistinguishable from the tea shot-hole borer (TSHB, *E. fornicatus* Eichhoff) (Eskalen et al. 2012, Mendel et al. 2012), but the PSHB is genetically distinct from TSHB (O'Donnell et al. 2015). Thus the common name polyphagous shot-hole borer (PSHB, Eskalen et al. 2013) has been applied to the beetle in California, which based on the sequences of multiple gene regions appears to be identical to the beetle found in Israel (O'Donnell et al. 2015). Beetles collected in the Los Angeles area also are genetically indistinguishable from Vietnam specimens (based on the sequences for the mitochondrial cytochrome oxidase I [COI], D2 domain of the 28S ribosomal subunit [28SD2] and elongation factor [*EF 1-α*]), which is thought to form part of the native range of the PSHB (R. Stouthamer unpubl).

Fusarium dieback spread in southern California from Los Angeles County to Orange, San Bernardino and Riverside counties within 2 y of first reported detection of this beetle (http://eskalenlab.ucr.edu/images/socalmap.jpg). In Dec 2013 a separate invasion was detected in a stand of California sycamore (*Platanus racemosa*) on a golf course in San Diego County, 60 miles south of the current infestation, and subsequently in commercial avocado groves 30 miles north of the sycamore stand. The beetle from LA (*Euwallacea* sp. #1) is morphologically indistinguishable but genetically distinct from the beetle in San Diego County (*Euwallacea* sp. #5), which carries its own novel species of *Fusarium* (O'Donnell et al. 2015). The infestation in LA also has spread from urban forests to commercial avocado groves, 10 Orange County parks and the Angeles National Forest (Pasadena Glen and Santa Anita canyons) on numerous hosts (see below). Therefore, Fusarium dieback is a complex that is of major concern in California for the avocado industry as well as landscapers and land managers in urban and wildland forests.

The rapid spread of the disease complex is attributed to the diverse range and quantity of reproductive hosts for the beetle that occur throughout multiple land-use areas in California. Reproductive hosts in California include the ubiquitous weed castor oil plant (*Ricinus communis* L.), avocado (*Persea americana*), 13 California native host species and seven commonly planted ornamentals in southern California (Eskalen et al. 2013), and the total number of confirmed reproductive hosts has increased 19–38 since 2012 (http://eskalenlab.ucr.edu/avocado.html). Hence an understanding of beetle-fungal-host interactions may provide clues to ultimately control the problem.

In contrast to bark beetles that are associated with phloem-inhabiting fungi (Six 2012), species of *Euwallacea* are wood-inhabiting ambrosia beetles and live in obligate mutualism with xylem-inhabiting ambrosia fungi. Conidia of the fungi are carried within the beetle mycangium (Fraedrich et al. 2008, Kasson et al. 2013) and are inoculated into the galleries by the adult females as they construct tunnels (Hulcr et al. 2007). Ambrosia fungi are continuously transmitted by females (rarely by males) to new tunnels during the excavation and before oviposition (Batra 1963, Fraedrich et al. 2008). This fungus colonizes the xylem and extracts and concentrates nutrients from it; it is the sole source of food for developing larvae and adult beetles (Harrington et al. 2010) as seen with *E. validus* (see SUPPLEMENTARY FIG. 2, Kasson et al. 2013) and their respective fusaria symbionts, AF-2 and AF-4. As the fungus colonizes the galleries it also spreads to the surrounding wood tissue, blocking vascular tissue in the xylem and impairing nutrient and water support (Hijii et al. 1991, Kendra et al. 2013). Trees with heavy fungal colonization and beetle attack exhibit wilting and leaf discoloration, which may lead to stem breakage or tree death (Mendel et al. 2012).

Symbionts of most previously studied ambrosia beetles are mostly ascomycete fungi in the orders Ophiostomatales (especially the genera *Dryadomyces*, *Ophiostoma*, *Raffaelea*) and Microascales (genera *Ambrosiella*, *Ceratocystis*, *Graphium*) (Norris 1968, Fraedrich et al. 2008, Alamouti et al. 2009, Baker and Harrington et al. 2010). Several ambrosia beetle associations with

Fusarium spp. (Hypocreales) (Gadd and Loos 1947, Norris and Baker 1967, Kessler 1974, Brayford 1987, Freeman et al. 2013, Kasson et al. 2013) and yeasts (Ascomycota: Saccharomycotina) such as *Ambrosiozyma*, *Ascoidea*, *Candida* and *Endomycopsis* (Batra 1963, Kinuura 1995) also have been reported. Recent findings indicate a strong association of *Fusarium* spp. with *Euwallacea* spp. on hosts in Israel, Australia, Sri Lanka and USA (California, Florida) (Kasson et al. 2013). Follow-up studies have revealed additional fusaria symbionts of *Euwallacea* spp. in India, Malaysia, California (San Diego) and Singapore (O'Donnell et al. 2015). Studies have suggested that, in addition to a putative member of the *Fusarium solani* species complex (reported as *F. solani*), a proposed symbiont of *Xyleborus ferrugineus* (Xyleborini), species of *Graphium* and *Acremonium* (formerly *Cephalosporium*), were essential for the beetle reproduction (Baker and Norris 1968). However, recent studies on *Xyleborus ferrugineus* suggest that oral mycangia contain a community dominated by Ophiostomatales symbionts (Kostovcik et al. 2014). Culture-dependant studies of fungi found in the heads of *Euwallacea validus* (a close relative of *E. fornicatus*) uncovered the presence of a second core community member, *Raffaelea subfusca*, found in near equal proportion to the fusaria symbiont (Kasson et al. 2013). These recent discoveries suggest that ambrosia symbionts often may occur in communities, but the occurrence of other fungal mycangial community members of the PSHB, in addition to *F. euwallaceae* has not yet been explored. The objectives of this study were to: (i) identify other common mycangial fungi, in addition to fusaria symbionts, carried by the PSHB in LA based on morphological characteristics and phylogenetic analysis of sequence data; (ii) quantify the occurrence of these fungi within female PSHB heads and the gallery walls produced by the beetle; (iii) determine pathogenicity of candidate mycangial fungi on two common reproductive hosts in California; and (iv) taxonomically assess and describe previously unknown fungal species that may be recovered.

MATERIALS AND METHODS

Specimen collections and fungal isolations.—Fungal species from both beetles and plant tissues infested by *Euwallacea* sp. #1 (PSHB from LA) were collected with methods of Eskalen et al. (2013). Collections were conducted within PSHB-infested stands in Pasadena, Whittier and Hacienda Heights in Los Angeles County, California. Three galleries and female adult beetles each per tree were sampled from three trees per host species including California sycamore (*Platanus racemosa*), box elder (*Acer negundo*), avocado (*Persea americana*) and castor oil plant (*R. communis*) (36 beetles and galleries total for each). Four beetles each were collected from galleries in castor oil plant and acacia (*Acacia auriculiformis*) in Vietnam. Fungal isolation and culture from USA and Vietnamese samples was performed at the University of California, Riverside, and the Forest Protection Research Centre in Hanoi, Vietnam, respectively.

To isolate fungi from symptomatic tissues, samples were flamed briefly and split in half. Pieces from the leading margin of clean necrotic tissue were excised with a sterile paring knife and plated onto potato dextrose agar (Difco) amended with 0.01% tetracycline hydrochloride (PDA-tet).

Female adult beetles blocking the entrance of mature galleries (i.e. galleries in which the beetle has returned to the entrance to maintain the fungal garden and protect the gallery from invaders after gallery excavation and egg deposition) were removed, and a cube surrounding the entry hole was cut to a depth of 2.54 cm for gallery sampling. The identity and abundance of fungal species within the pre-oral mycangia of freshly collected female PSHB specimens were determined by culturing a solution of macerated aseptically dissected beetle heads, where mycangia had been confirmed (Eskalen et al. 2013). Tubes containing preserved beetles were vortexed 10 s followed by three serial washings with sterilized distilled water. The heads were aseptically removed from the beetles and macerated in a 1.5 mL tube containing 200 µL sterile water with a sterile pestle. A 50 µL suspension was spread onto a PDA-tet media after vortexing the tubes 10 s. To determine identity and abundance of fungal species within gallery walls, tissues lining the galleries were scraped with a sterilized needle, transferred to a small screw-cap bottle containing 100 µL sterilized distilled water, shaken thoroughly, and a 10 µL suspension was spread onto a PDA-tet medium.

Fungi were identified with morphological and molecular techniques (see below). Colony forming units (CFUs) that were consistent in color, growth rate and texture were subcultured for individual identification and counted on all plates after 3 d to quantify the propagules present within the heads of each individual beetle. Relative abundances of fungal species within each beetle head or gallery wall was determined based on the number of CFUs per microliter for each species, divided by the total CFUs of all fungal species combined.

Morphological characterization.—The putative identities of isolates recovered from trees and beetle specimens were based on morphology. Fungi were propagated by growing at least nine isolates each on either oatmeal agar (OMA, Difco) or PDA-tet to assess spore morphology following methods of Paciura et al. (2010). The length and width of 50 conidia per isolate were measured with a compound microscope (Olympus BX40 with a Leica DFC420 camera) and the SPOT Imaging software (Diagnostic Instruments, Sterling Heights, Michigan). Summary statistics (minimum, mode, maximum, mean, standard deviation) for lengths and widths of conidia were calculated with SAS 9.3 (SAS Institute, Cary, North Carolina). In addition, four isolates for each fungal morphotype detected (Morphotype 1: UCR1780, UCR1781, UCR1852, UCR1876; Morphotype 2: UCR2980, UCR2981, UCR2067, UCR 2975; UCR 2982, UCR 2984, UCR 2991, UCR 2996) were grown on PDA-tet and incubated at 5, 10, 15, 20, 25, 30, 35 and 40 C to determine the effect of

TABLE I. Frequency (%) of *Fusarium euwallaceae*, *Paracremonium pembeum* and *Graphium euwallaceae* recovered from 36 *Euwallacea* sp. heads and galleries collected from *Platanus racemosa*, *Acer negundo*, *Persea americana* and *Ricinus communis* in the Los Angeles Basin

Fungus	*Fusarium euwallaceae*		*Acremonium pembeum*		*Graphium euwallaceae*	
Host/sample	Heads	Galleries	Heads	Galleries	Heads	Galleries
Platanus racemosa	100	100	14.3	22.2	57.1	66.7
Acer negundo	77.8	75	33.3	100	22.2	37.5
Persea americana	77.8	77.8	22.2	66.7	44.4	77.8
Ricinus communis	90.9	60	90.9	100	45.5	50

temperature on colony growth. Colony diameter was measured after 14 d incubation. The experiment was conducted twice.

Isolates from other *Euwallacea* spp. with similar fungal morphotypes collected from other areas also were included for comparative purposes (SUPPLEMENTARY TABLES I–IV).

DNA extraction, polymerase chain reaction amplification and phylogenetic analyses.—In addition to morphological studies species identification was further refined through BLASTn queries of sequence data from a portion of the nuclear ribosomal RNA gene repeat (rDNA) comprising the internal transcribed spacer region (ITS)in GenBank. When BLASTn queries did not identify any closely related species with high sequence identity (<99% match with known sequences in GenBank), sequences of isolates were compared to those of previous studies with multigene phylogenetic analyses (SUPPLEMENTARY TABLES I, II). Genomic DNA was extracted from pure cultures of each isolate following the method of Cenis (1992). Portions of the internal transcribed spacer (ITS1-5.8S-ITS2) region, translation elongation factor 1-α (*EF1-α*) gene, calmodulin (*cmdA*), large subunit of the ATP citrate lyase (*acl1*), β-tubulin (*tub2*), RNA polymerase II second largest subunit (*rpb2*) and either the 28S the large subunit rDNA or 18S the small subunit rDNA (depending on the fungal species) were chosen for multilocus sequence typing. Oligonucleotide primers ITS4 and ITS5, EF1F and EF2R, CAL-228F and CAL2Rd, acl1-230up and acl1-1220low, T1 and CYLTUB1R, RPB2-5F2 and RPB2-7cR, V9G and LR5 and NS1 and NS8 were used to amplify the ITS1-5.8S-ITS2 (ITS) (White et al. 1990), Gräfenhan *EF1-α* (Jacobs et al. 2004), *cmdA* (Carbone and Kohn 1999, Groenewald et al. 2013), *acl1*, *tub2* (O'Donnell and Cigelnik 1997, Crous et al. 2004), *rpb2* (O'Donnell et al. 2007), 28S (Vilgalys and Hester 1990, Hoog and Gerrits van den Ende 1998) and 18S (White et al. 1990) regions, respectively. PCR reaction mixtures for all genes consisted of 2.5 μL 10 × reaction buffer, 0.5 μL dNTPs (200 μM), 0.5 μL each primer (0.2 μM), 0.25 μL standard Taq polymerase (New England Biolabs) and 1.5 μL DNA for a total of 25 μL reaction volume. PCR reactions were conducted in a thermal-cycler (Bio-Rad Laboratories) with published cycling conditions (White et al. 1990, Jacobs et al. 2004). Amplification products were separated by electrophoresis in 1.5% agarose gels in 0.5 × Tris-boric acid-EDTA buffer and visualized under UV light after staining with SYBR Green (Invitrogen). PCR products were purified with a GeneJET Kit (Thermo Scientific). ITS, *EF1-α*, 28S and 18S regions were sequenced in both directions at the Institute for Integrative Genome Biology, University of California, Riverside.

Phylogenetic analyses were conducted with MEGA 5.0 (Tamura et al. 2007) using maximum likelihood, neighbor-joining and maximum parsimony. Alignment gaps were treated as missing data. Maximum-likelihood and parsimony heuristic searches using the CLOSE-NEIGHBOR INTERCHANGE (CNI) BRANCH SWAPPING option were conducted. Bootstrap values were calculated with the Kimura 2-parameter model with 1000 replicates and 100 random sequence additions to test branch support. Sequences for each species recovered in this study were compared with those from reliable sources available in GenBank, with closely related taxa used as outgroups (SUPPLEMENTARY TABLES I, II). Complete sequence alignments for all isolates are available in TreeBASE (submission IDs 17955, 17954).

Pathogenicity tests.—Two isolates each of the species most commonly associated with symptomatic plant tissue, beetle galleries and specimens of *Euwallacea* sp. from the Los Angeles Basin were used in comparative pathogenicity testing. Representative isolates included UCR2975 and UCR2980 for fungal morphotype 1 and UCR2982 and UCR2993 for fungal morphotype 2. Two isolates of *Fusarium euwallaceae* (UCR1854, UCR3200) recovered from avocado and box elder served as positive controls. Isolates were grown on PDA-tet 3–5 d before inoculation of 30 cm detached healthy 2.54 cm woody shoots of box elder and avocado collected from landscape trees and the Agricultural Operations Field Station at the University of California, Riverside. Xylem tissue was excised from the center of each shoot with a 3 mm cork borer. Following removal a 3 mm diam agar plug was cut from the leading edge of each growing culture and placed mycelium-side down onto the freshly wounded tissue; the inoculated area was covered with parafilm. Inoculated shoots and controls (inoculated with sterile PDA-tet plugs) were covered with wax at their ends to prevent desiccation and were incubated at 25 C in moist chambers for 4 wk. After incubation the extent of vascular discoloration and recovery of fungal isolates 2.0 cm above the inoculation points were determined. The experiment was arranged in a randomized design with five replications per isolate and conducted twice.

TABLE II. Number of occurrences and co-occurrences of *F. euwallaceae* (F), *Graphium euwallaceae* (G), and *Paracremonium pembeum* (P) within 36 heads and gallery walls of *Euwallacea* sp. collected from four hosts in the Los Angeles Basin

Fungal occurrence	Heads	Gallery walls
FGP	5	11
FG	8	8
FP	8	7
GP	1	3
F	11	2
G	0	0
P	12	5

Statistical analyses.—Differences among spore sizes were tested by analysis of variance (ANOVA) in SAS 9.4 (SAS Institute Inc., Cary, North Carolina), and means were compared with Fisher's protected least significant difference (LSD) at $\alpha = 0.05$. A nonparametric bootstrapping model was used to compare relative abundance of fungal species recovered from beetle heads and gallery walls between and within hosts. Differences in means were compared with the bootstrap procedure $\alpha = 0.05$.

Radial growth values of pure cultures after 14 d at eight temperatures were fitted to a Weibull five-parameter regression curve with a probability density function (Miller et al. 2003) in SigmaPlot 11 (Systat Software Inc., San Jose, California).

Lesion-length data from repeated experiments were tested for homogeneity of error variance with Bartlett's test in SAS. Differences in mean lesion length among isolates and treatments were tested by analysis of variance (ANOVA) in PROC GLM of SAS if the homogeneity of variance testing was not significant ($P > 0.05$). Means lesion lengths among isolates were compared with the LSD test at $\alpha = 0.05$.

RESULTS

Fungal recovery, frequency and relative abundance within beetle heads and gallery walls.—Based on morphological characterization (see below) and BLASTn query comparisons of the ITS sequence data in the present study to other isolates in GenBank, two undescribed fungal species were recovered consistently from beetles from the Los Angeles Basin in California (LA): *Graphium* sp. Corda (family Microascaceae) and *Paracremonium* sp. L. Lombard & Crous (family Nectriaceae). *Fusarium euwallaceae*, *Paracremonium pembeum* sp. nov. and *Graphium euwallaceae* sp. nov. (see phylogenetic analyses and descriptions below) were recovered from female PSHB heads and gallery walls collected from infested California sycamore, box elder, avocado and castor oil plant throughout LA. *Fusarium euwallaceae*, *G. euwallaceae*, *G. carbonarium*, *Graphium* sp. III and *Paracremonium* sp. I (see phylogenetic analyses below) also were recovered from female PSHB heads and gallery walls collected from castor oil plant and acacia in Vietnam. Colonies of fungal species co-occurred on PDA plates without inhibition or parasitism and grew at different rates (FIG. 1). However, the incidence of co-occurrence, frequency and relative abundances of each species varied across the 36 beetle head and 36 gallery wall samples collected from the four different host plant species (TABLES I, II; FIGS. 2–4). *F. euwallaceae*, *G. euwallaceae* and *P. pembeum* were

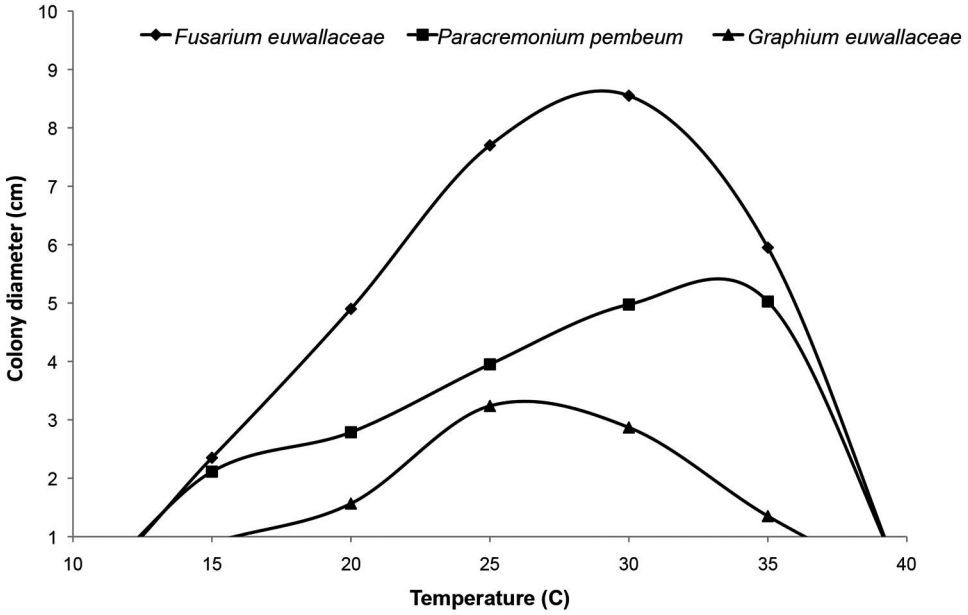


FIG. 1. Optimum temperature of *Fusarium euwallaceae*, *Graphium euwallaceae* and *Paracremonium pembeum*. Colony diameter (cm) on PDA in 9 cm diam Petri dishes was recorded 14 d after plating.

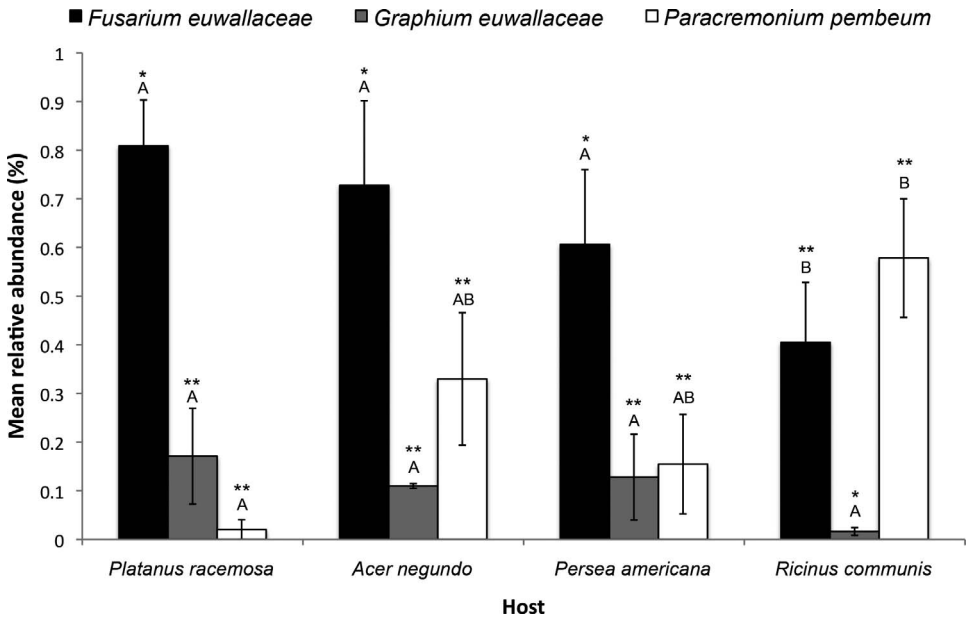


FIG. 2. Mean relative abundance of colony forming units of three species recovered from the heads of *Euwallacea* sp. collected from *Platanus racemosa, Acer negundo, Persea americana* and *Ricinus communis*. Vertical lines represent standard error according to the bootstrap procedure test for differences in means at $\alpha = 0.05$. Relative abundances of fungal species that share the same letters between respective hosts are not significantly different. Relative abundances of fungal species that share the same number of asterisks within each host are not significantly different.

recovered in LA from the heads of 31, 15 and 16 beetles and 28, 22 and 26 gallery walls, respectively. Occurrences and co-occurrences of all three fungi varied (TABLE II). All three fungi co-occurred within 11 gallery walls and five PSHB heads, and *F. euwallaceae* co-occurred with either *P. pembeum* or *G. euwallaceae* equally between the two sample types (TABLE II). *F. euwallaceae* occurred alone in more beetle heads than gallery walls, and *G. euwallaceae* never occurred alone in either.

F. euwallaceae was recovered from beetle heads at higher frequencies and abundances in nearly all tree hosts from which beetles were collected. Relative abundances of *F. euwallaceae* within beetle heads from each host except castor oil plant were greater than *P. pembeum* and *G. euwallaceae* ($P < 0.0142$) (FIG. 2). For beetles collected from castor oil plant *F. euwallaceae* and *P. pembeum* were relatively more abundant in beetle heads than *G. euwallaceae* ($P = 0.0022$) and were not significantly different. Among hosts relative abundance of *P. pembeum* in beetle heads collected from castor oil plant was greater than those collected from sycamore ($P < 0.0083$) and relative abundance of *F. euwallaceae* within beetle heads collected from sycamore was greater than those collected from castor oil plant ($P < 0.0475$) (FIG. 2).

Within hosts *P. pembeum* was relatively more abundant than other fungal species within the gallery walls of box elder and castor oil plant (FIG. 3). Among hosts *F. euwallaceae* and *G. euwallaceae* were relatively more abundant within the gallery walls of sycamore and avocado, respectively, ($P < 0.0011$, $P < 0.0025$) and *P. pembeum* was relatively more abundant in box elder, avocado and castor oil plant ($P < 0.0001$). In all hosts *F. euwallaceae* was relatively more abundant within beetle heads than gallery walls ($P < 0.0001$) (FIG. 4).

Morphological characterization.—Thirteen *G. euwallaceae* isolates in this study were used to assess colony and propagule morphology (FIGS. 5, 6). Isolates produced synnema and annellidic conidiogenensis characteristic of taxa in the genus *Graphium* (Okada et al. 1998, 2000). Colony diameters were 2.9–3.6 and 3.7–4.4 cm on PDA after 14 d in the dark, respectively, at 25 C and 30 C; the optimum growth temperature was 25–30 C, but growth occurred at 35 C (FIG. 1). Young colonies (< a week) were whitish on both OMA and PDA, becoming dark grayish olivaceous with whitish peripheries with age (> a week) and whitish patches on PDA (FIG. 5a–d). Aerial mycelium on PDA was hyaline and septate but not constricted at the septa, 2.0–3.5 μm wide; on OMA colonies were flat. Colony on the reverse plate was dark gray for both media in older cultures. Similar to *G. fabiforme* (Cruywagen et al. 2010), *G. basitruncatum* and *G. carbonarium* (Paciura et al. 2010), which produce more than one type of conidia, isolates in the present study produced two types of aseptate conidia. Unlike *G. fabiforme*, which

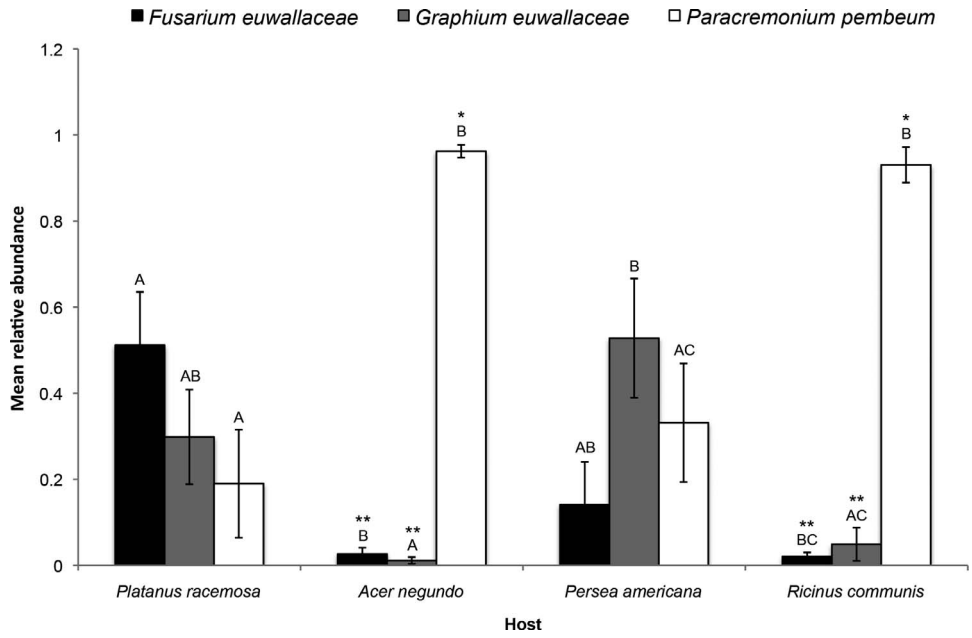


FIG. 3. Mean relative abundance of colony forming units of three species recovered from the heads of *Euwallacea* sp. collected from *Platanus racemosa, Acer negundo, Persea americana* and *Ricinus communis*. Vertical lines represent standard error according to the bootstrap procedure test for differences in means at $\alpha = 0.05$. Relative abundances of fungal species that share the same letters between respective hosts are not significantly different. Relative abundances of fungal species that share the same number of asterisks within each host are not significantly different.

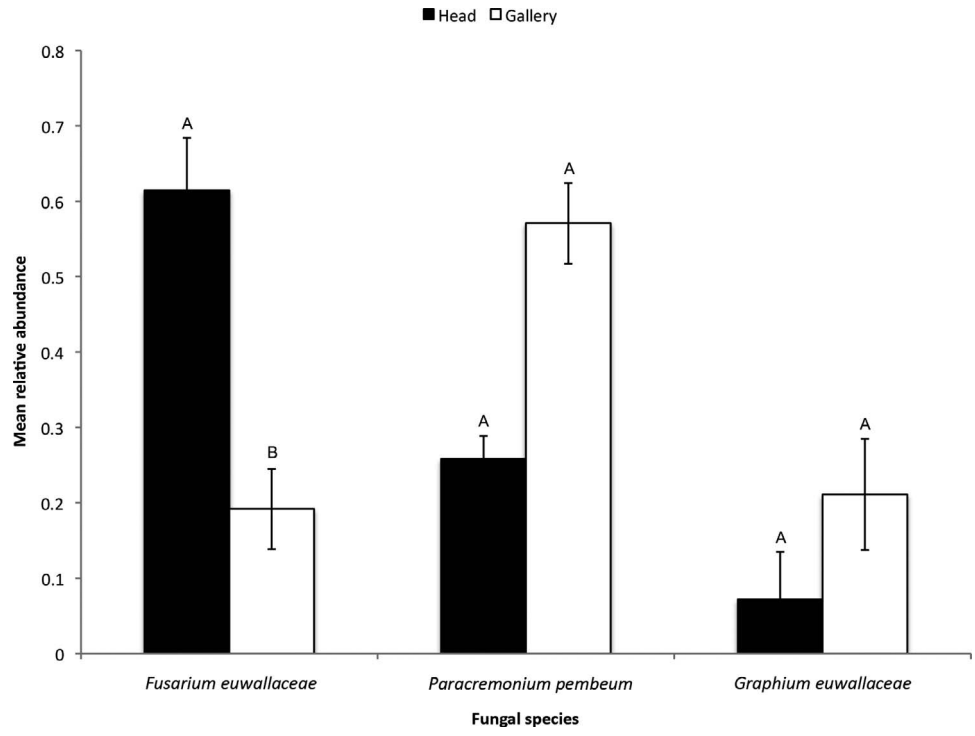
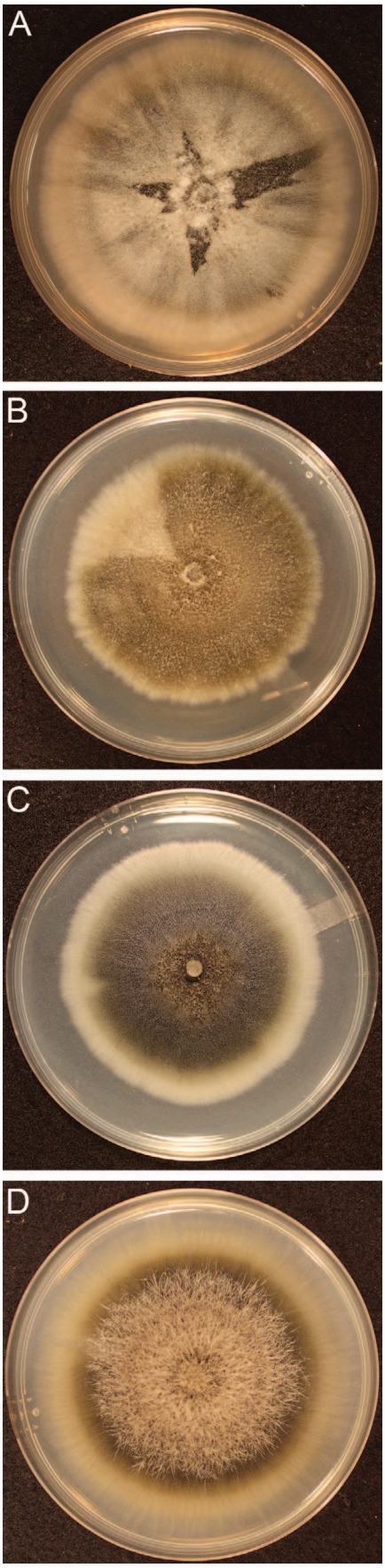


FIG. 4. Mean relative abundance of colony forming units of three species recovered from the heads and gallery walls of *Euwallacea* sp. collected from all hosts. Vertical lines represent standard error according to the bootstrap procedure test for differences in means at $\alpha = 0.05$. Relative abundances that share the same letters within each fungal species are not significantly different.



produced cylindrical to obovoid and reniform conidia (Cruywagen et al. 2010), conidia of isolates from the present study were similar to those of *G. basitruncatum* and *G. carbonarium* (Paciura et al. 2010): cylindrical with truncated bases and obovoid. Both conidial types were shorter and narrower in *G. euwallaceae* isolates from California in the present study compared to *G. basitruncatum* and *G. carbonarium*. The first conidial type of the California isolates was longer than those in isolates from Vietnam and thinner than those from Thailand and Pennsylvania. The second conidial type of the California isolates was slightly shorter and thinner than those in isolates from Vietnam. Conidial measurements obtained for the *Graphium* species are provided (SUPPLEMENTARY TABLE III) together with reported measurements (Jacobs et al. 2003, Cruywagen et al. 2010, Paciura et al. 2010).

Three *Paracremonium* sp. I isolates from Vietnam and 10 *P. pembeum* isolates from California were compared to those of *Xenoacremonium recifei* L. Lombard & Crous, *Paracremonium contagium* L. Lombard Crous and *Paracremonium inflatum* L. Lombard & Crous (Summerbell et al. 2011, Lombard et al. 2015) from the Centraalbureau voor Schimmelcultures (CBS) culture collection in the Netherlands (SUPPLEMENTARY TABLE IV). Optimal growth temperature of the California isolates of *P. pembeum* was 30–35 C after 14 d incubation in the dark and no growth occurred at 40 C (FIG. 1). Colonies on PDA had regular margins, were pale pink (paler at the peripheries), with septate hyphae that were flat to sparsely aerial, 2.0–2.7 µm wide; colonies on malt extract agar were flat and white. Conidiophores of the isolates from California consisted of single, tapered 13.9–41.4 µm (28.2 ± 1.3 µm) long phialides or 2–3 phialides borne on a stipe arising from vegetative and aerial hyphae. Conidiophores of the Vietnamese isolates consisted of simple phialides or conidiophores with branches giving rise to 1–2 phialides and/or metulae giving rise to 2–3 phialides. Conidiophores radiating from sterile coils and inconspicuously swollen septa in the hyphae (Lombard et al. 2015) were not observed in the isolates from California or Vietnam. Conidia of the isolates from California, Vietnam and of *X. recifei* (CBS 137.35, CBS 188.82, CBS 220.84) were hyaline, aseptate and produced in slimy heads (FIG. 7). Globose to ellipsoidal, hyaline, thick-walled chlamydospores arranged in intercalary chains were observed on the California isolates (FIG. 7). Length, width and length-width ratio were compared among all isolates, and while there were significant differences

←

FIG. 5. a. Upper surface of 4 wk old cultures on PDA of *Graphium euwallaceae*. b. *Graphium* sp. II (from Thailand). c. *Graphium* sp. III (from Vietnam). d. *Graphium* sp. I (from Pennsylvania).

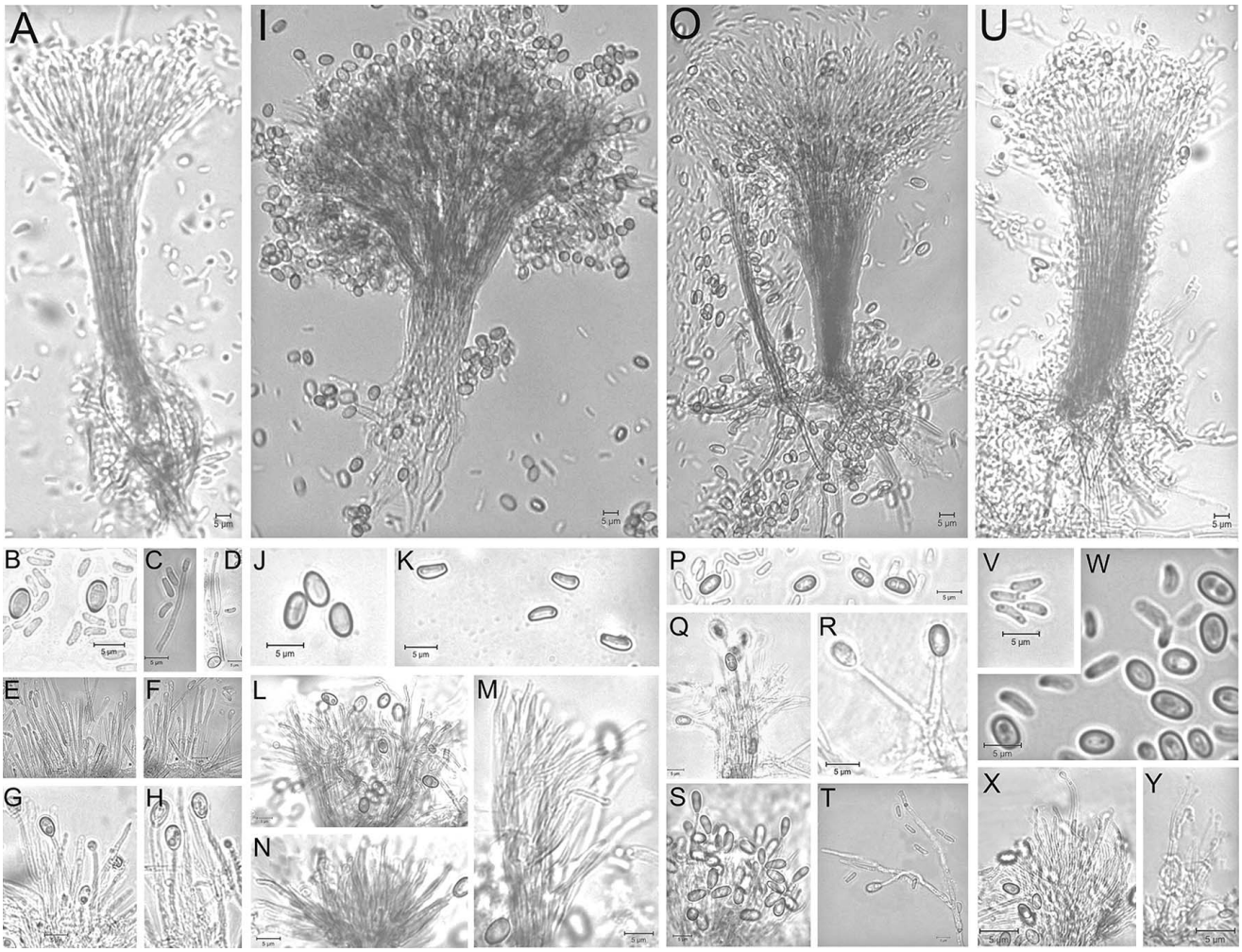


FIG. 6. *Graphium euwallaceae*. a. Synnema. b. Thin and round conidia. c. Conidiogenous cells. d–h. *Graphium* sp. III (from Vietnam). i. Synnema. j. Round conidia. k. Thin conidia. l–n. Conidiogenous cells. *Graphium* sp. I (from Pennsylvania). o. Synnema. p. Thin and round conidia. q–t. Conidiogenous cells. u–y. *Graphium* sp. II (from Thailand). u. Synnema. v. Thin conidia. w. Thin and round conidia. x, y. Conidiogenous cells.

between different pairs of isolates no significant difference was observed between isolates grouped by location.

Phylogenetic analysis.—Phylogenetic analysis of the combined ITS and *EF1-α* sequences of isolates from California and Pennsylvania (USA), Vietnam and Thailand putatively identified as *Graphium* and sequences from GenBank that represent known species of *Graphium* resolved four major lineages with *Geosmithia pallida* (CCF 4319) and *Ophiostoma ulmi* (Q412T-0) as outgroup clade (FIG. 8). Phylogenetic analysis of the combined ITS, *EF1-α* and 18S sequences yielded the same topology with fewer taxa (data not shown). Maximum-likelihood, parsimony and neighbor-joining analysis yielded the same topologies and similar bootstrap values.

The first clade (100% bootstrap support) corresponded to groups that produce two types of conidia (cylindrical, tear-drop). This clade resolved further into four lineages: one that contained strains from California and a single strain from Vietnam (98% bootstrap support); *Graphium carbonarium*, which also contained isolates from Vietnam; *Graphium* sp. I, II and III, which contained strains from Pennsylvania, Thailand and Vietnam (98%, 77%, 99% bootstrap support); and *Graphium basitruncatum*. Isolates from California formed a highly supported clade that was different from lineages recovered from *Euwallacea validus* and *Euwallacea fornicates*, respectively, in Pennsylvania and Thailand (CABI, 2015). Sequences from isolates recovered from the heads of female PSHB beetles in Vietnam resolved three different species: *G. carbonarium* and two previously undescribed species, one of which is from California.

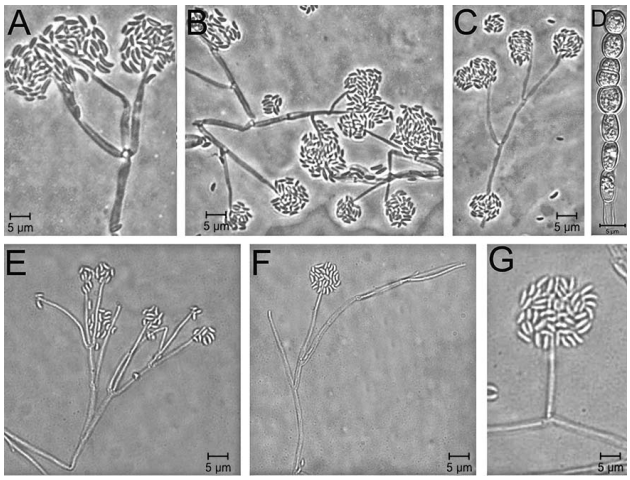


FIG. 7. a–c. Conidial heads of *Paracremonium pembeum* on simple phialides or conidiophores bearing 1–3 phialides. d. Chlamydospores. e–f. Conidiophores of *Paracremonium* sp. I (from Vietnam) consisting of branches borne on a stipe, giving rise to 1–2 phialides and/or metulae, giving rise to 2–3 phialides (e–f) or simple phialides (g).

The second clade (100% bootstrap support) contained lineages representing *Graphium penicillioides* (sensu stricto); a third clade (100% bootstrap support) resolved further into *Graphium madagascariense* and *Graphium adansoniae* (100% bootstrap support each). The fourth clade (85% bootstrap support) contained two subclades: one containing *Graphium fabiforme* (100% bootstrap support); a second containing *Graphium fimbriisporum* (100% bootstrap support) and another subclade (99% bootstrap support) that resolved further into *Graphium pseudormiticum* and *Graphium laricis* (99% bootstrap support each).

Phylogenetic analysis of the combined 28S, ITS *EF1-α*, *tub2*, *rpb2*, *cmdA* and *acl1* sequences of isolates from Vietnam and the Los Angeles Basin putatively identified as *Paracremonium* sp., in comparison with GenBank sequences and sequences derived from CBS cultures that represent known species in the Nectriaceae family, revealed that the isolates recovered from PSHB heads collected from tree hosts in California and Vietnam resolved in a lineage closely related to *P. inflatum* (100% bootstrap support) that resolved further into three distinct groups (FIG. 9). As with separate analyses of the 28S, ITS, *EF1-α*, *cmdA*, *acl1*, *tub2* and *rpb2* genes isolates from California were distinct from *P. contagium*. *Paracremonium* isolates were distinct from *X. recifei* and other species in the Nectriaceae, with *Tilachlidium brachiatum* L. Lombard & Crous (family Tilachlidiaceae) (Lombard et al. 2015) as outgroup. Maximum-likelihood, parsimony and neighbor-joining analyses yielded similar topologies.

Pathogenicity tests.—All isolates tested colonized healthy inoculated excised stems of avocado and box elder (FIG. 10). Re-isolation (re-recovered in 80–100% inoculated plants) confirmed the same species as those used in the inoculations based on colony morphology and sequence data. The average distance of growth in wood was not significantly different between trials ($P > 0.05$), and thus the data were pooled. Average distance of growth was significantly different between fungal inoculation treatments and controls ($P < 0.05$), while the magnitude of colonization varied by fungal species (FIG. 10). Lesions produced by isolates of the three fungi did not significantly differ in length on box elder ($P = 0.6302$), but on avocado the *P. pembeum* produced longer lesions than either *F. euwallaceae* or *G. euwallaceae* ($P = 0.0212$) (FIG. 10).

TAXONOMY

Graphium euwallaceae M. Twizeyimana, S.C. Lynch, A. Eskalen, sp. nov. FIGS. 1, 5, 6
MycoBank MB813074

Typification: UNITED STATES. CALIFORNIA. Hacienda Heights, Los Angeles County. Living *Euwallacea* sp., collected from galleries of infested Hass avocado tree (*Persea americana* Mill.), 5 Aug 2012, *A. Eskalen*, (**holotype** BPI 893108). Ex-type cultures CBS 140035 = UCR2980.

Etymology: Derived from the generic name of the host ambrosia beetle from which it was recovered, the polyphagous shot hole borer (*Euwallacea* sp.).

No teleomorph observed. Synnemata arising singly or in groups, consisting of a stripe of stacked parallel hyphae, a divergent capitulum of conidiogenous cells and mucilaginous mass of conidia. Synnematal stipe, olivaceous gray at the base, becoming hyaline toward the apex, 155 ± 35 µm long, 36 ± 3.5 µm wide at the midregion, 44 ± 4.5 µm wide at the apex. Rhizoid-like hyphae at the base abundant. Conidiophores produced in the divergent capitulum with two to four series of branches, with 3–5 metulae or conidiogenous cells per branch point, metulae or conidiogenous cells 15 ± 3.5 µm long and 1.0–3.0 µm wide, conidium development annellidic, or sometimes giving the impression of sympodial proliferation. Two types of conidia, (i) hyaline, cylindrical conidia with a conspicuous basal frill 4.0–6.0 × 1.0–2.0 µm (4.5 ± 0.0 × 1.3 ± 0.0) µm, (ii) obovoid, thick-walled, 5.0–6.0 × 3.0–4.0 µm (5.0 ± 0.0 × 3.0 ± 0.0); conidia aseptate (single-celled) and formed in hyaline chain-like masses at the apices of conidiophores, bright transparent white when young, turning dark grayish olivaceous with age.

Hosts range: Acer negundo, Persea americana, Platanus racemosa, Ricinus communis, Euwallacea sp.

Fungal distribution: California and Vietnam

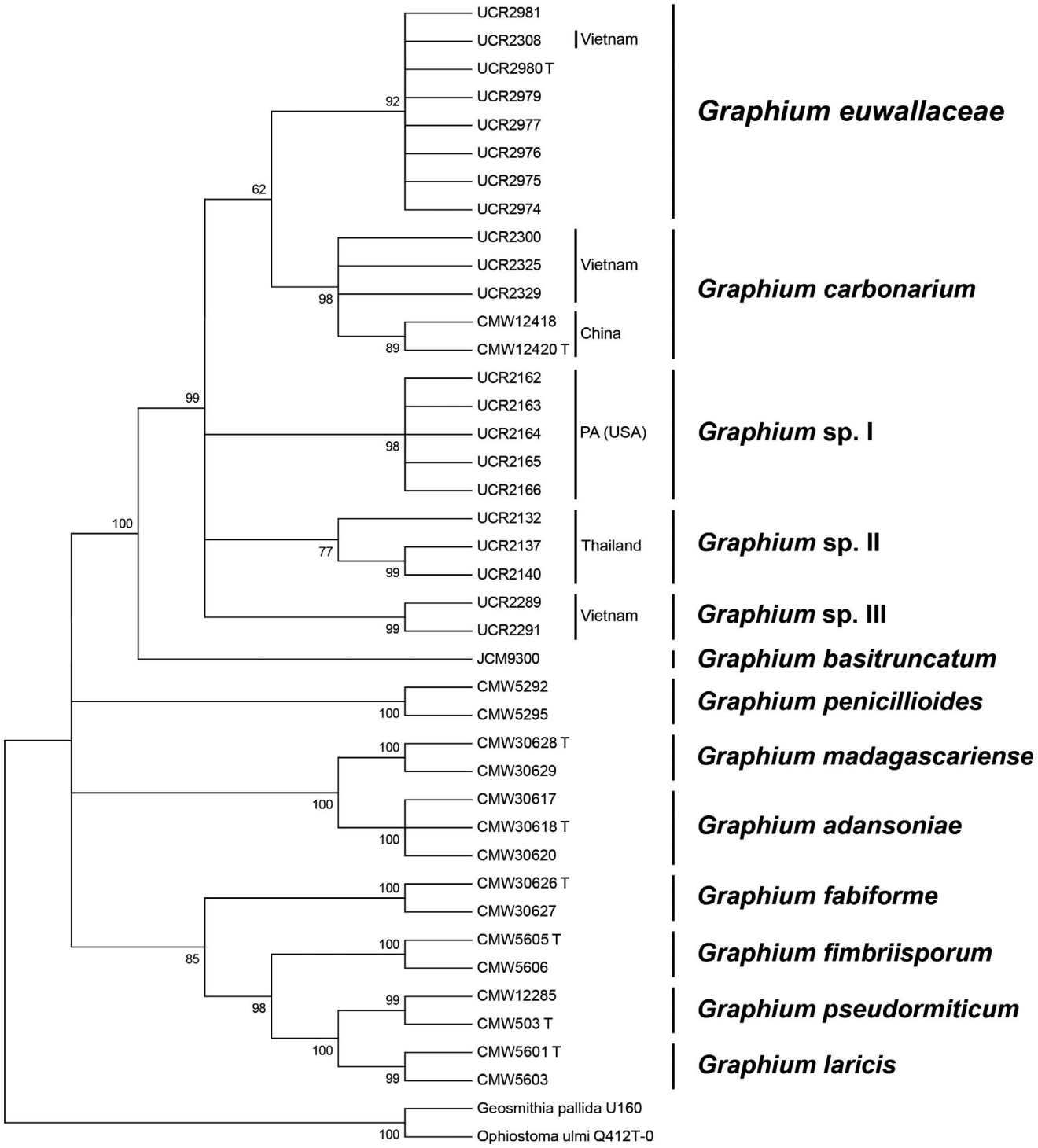


FIG. 8. Maximum-likelihood tree derived from analysis of combined internal transcribed spacer (ITS) and elongation factor (*EF1-α*) sequences using Kimura two-parameter model for *Graphium* species. Bootstrap support values higher than 50% from 1000 replicates are at the nodes. Origins (Vietnam, China, Pennsylvania, Thailand) are identified for *Graphium euwallaceae*, *G. carbonarium* and *Graphium* sp. I, II, III. Ex-type strains (T) are identified.

Additional specimens examined: VIETNAM. HANOI. Gallery wall excavated by *Euwallacea* sp. on *Acacia auriculiformis*, 2013, A. Eskalen, R. Stouthamer, T. Pham, *UCR2308*, *CBS 140667* (culture); UNITED STATES. CALIFORNIA. Huntington Botanical Gardens, Los Angeles County. Head of *Euwallacea* sp., collected from galleries of infested *Ricinus communis*, 2013, A. Eskalen, *UCR2974*, *CBS 140108* (culture); UNITED STATES. CALIFORNIA. Huntington Botanical

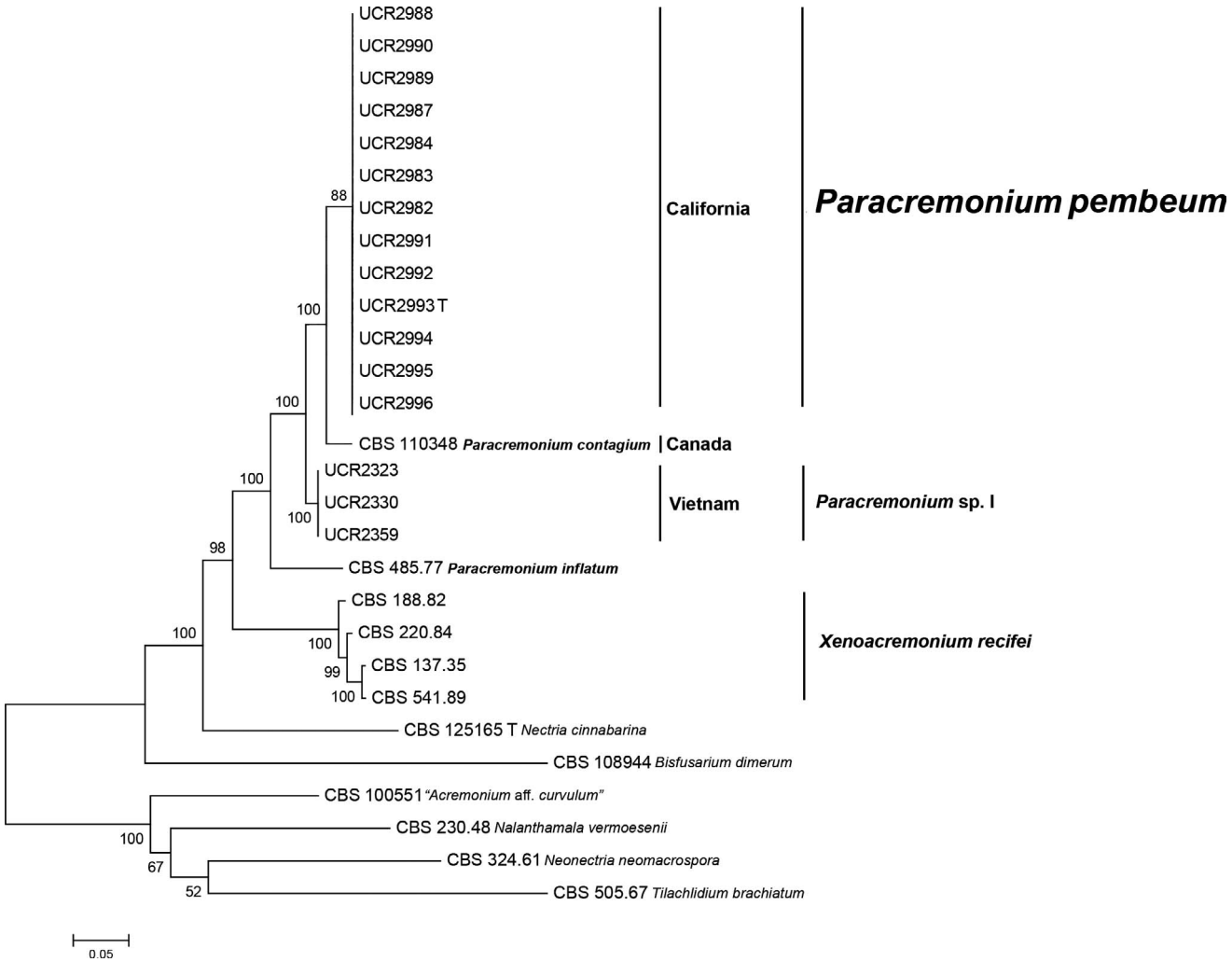


FIG. 9. Maximum-likelihood tree derived from analysis of the combined large subunit (28S), internal transcribed spacer (ITS), elongation factor (*EF1-α*), calmodulin (*cmdA*), large subunit of the ATP citrate lyase (*acl1*), β-tubulin (*tub2*) and RNA polymerase II second largest subunit (*rpb2*) rDNA sequences using Kimura two-parameter model for *Paracremonium* and related species in the Nectriaceae. Bootstrap support values higher than 50% from 1000 replicates are at the nodes. Origins for *P. pembeum* (California and Canada) and *Paracremonium* sp. I (Vietnam) are ex-type strains (T).

Gardens, Los Angeles County. Larvae of *Euwallacea* sp., collected from galleries of infested *Ricinus communis*, 2013, A. Eskalen, *UCR2976*, *CBS 140110* (culture); UNITED STATES. CALIFORNIA. Huntington Botanical Gardens, Los Angeles County. Abdomen of *Euwallacea* sp., collected from galleries of infested *Acer negundo*, 2013, A. Eskalen, *UCR2975*, *CBS 140109* (culture); UNITED STATES. CALIFORNIA. Huntington Botanical Gardens, Los Angeles County. Abdomen of *Euwallacea* sp., collected from galleries of infested *Acacia floribunda*, 2013, A. Eskalen, *UCR2977*, *CBS 140034* (culture); UNITED STATES. CALIFORNIA. Huntington Botanical Gardens, Los Angeles County. Abdomen of *Euwallacea* sp., collected from galleries of infested *Erythrina atitlanensis*, 2013, A. Eskalen, *UCR2978*, *CBS 140111* (culture); UNITED STATES. CALIFORNIA. Huntington Botanical Gardens, Los Angeles County. Head of *Euwallacea* sp., collected from galleries of infested *Quercus agrifolia*, 2013, A. Eskalen, *UCR2979*, *CBS 140112* (culture); UNITED STATES. CALIFORNIA. Huntington Botanical Gardens, Los Angeles County. Abdomen of *Euwallacea* sp., collected from galleries of infested *Persea americana*, 2013, A. Eskalen, *UCR2981*, *CBS 140113* (culture).

Comments: Phylogenetic analyses reveal that *G. euwallaceae*, which was recovered from the heads of *Euwallacea* sp. *#1* collected from avocado, California sycamore, box elder and castor oil plant throughout the Los Angeles Basin, is distinct but closely related to *G. carbonarium* and *G. basitruncatum* and three undescribed species of *Graphium* from Pennsylvania, Vietnam and Thailand. *G. euwallaceae*, *G. carbonarium* and *G. basitruncatum* produce two types of aseptate conidia described herein. The first conidial type was

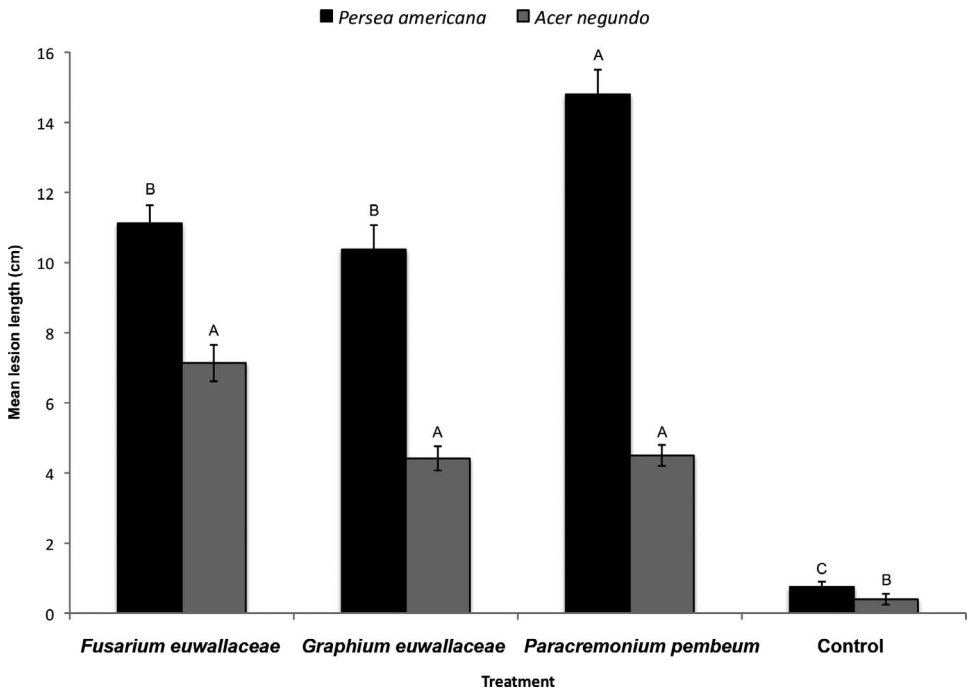


FIG. 10. Mean lesion length on avocado and box elder shoots inoculated with isolates of *F. euwallaceae*, *G. euwallaceae* and *Paracremonium pembeum*. Vertical lines represent standard error of the mean according to Fisher protected least significant difference (LSD) mean separation test at $\alpha = 0.05$. Lesion lengths that share the same letters for each host species are not significantly different.

longer in *G. euwallaceae* than those in isolates from Vietnam and thinner than those from Thailand and Pennsylvania; the second conidial type was slightly shorter and thinner in *G. euwallaceae* than those in isolates from Vietnam. The first conidial type was slightly shorter and narrower in *G. euwallaceae* compared to *G. basitruncatum* and *G. carbonarium*; the second conidial type was shorter and narrower in *G. euwallaceae* compared to *G. carbonarium* and *G. basitruncatum*. The dark grayish olivaceous of *G. euwallaceae* colony in old culture on OMA is similar to that of *G. carbonarium* and *G. basitruncatum* (Paciura et al. 2010) and *G. fabiforme* (Cruywagen et al. 2010) but is different from that of *G. madagascariense* and *G. adansoniae*, which are hazel (Cruywagen et al. 2010) or *G. laricis* with cinnamon brown (Jacobs et al 2003) on oatmeal agar.

Paracremonium pembeum S.C. Lynch, A. Eskalen, sp. nov. FIGS. 1, 7
MycoBank MB813071

Typification: UNITED STATES. CALIFORNIA. Pasadena, Los Angeles County. Head of *Euwallacea* sp., collected from galleries of infested *Ricinus communis*, 2013, *D.H. Wang/F. Na/A. Eskalen*, (**holotype** BPI 893107). Ex-type cultures CBS 140042 = UCR2993.

Etymology: Derived from the pink mycelium formed in culture on PDA; the root pembe signifies pink in Turkish.

No teleomorph observed. Colonies pale pink on PDA with regular margins, consisting of septate hyphae 2.2 ± 0.2 µm wide that are flat to sparsely aerial growing 0.5–1.0 mm above the agar; colonies on malt extract agar flat and white. Mycelial growth reaching 5 cm after 14 d at 30 C (optimum temperature); does not grow at 5, 10 or 40 C. Conidiophores consisting of single, tapered (14.0–)31.0–41.5 µm (28.2 ± 1.3 µm) long, phialides or 2–3 phialides borne on a stipe arising from vegetative and aerial hyphae. Conidia hyaline, aseptate aggregating in slimy heads, smooth-walled, sickle-to-torpedo shaped, (3.0–)4.5–7.0 × (0.5–)1.0–2.5 µm (4.8 ± 0.0 µm × 1.4 ± 0.0 µm). Globose to ellipsoidal, hyaline, thick walled chlamydospores arranged in intercalary chains observed.

Hosts: Acer negundo, Persea americana, Platanus racemosa, Ricinus communis, Euwallacea sp.

Fungal distribution: Tree hosts and the heads of *Euwallacea* sp. throughout Fusarium dieback-infested areas in southern California.

Additional specimens examined: UNITED STATES. CALIFORNIA. Huntington Botanical Gardens, Los Angeles County. Head of *Euwallacea* sp., collected from galleries of infested *Acer negundo*, 2013, D.H. Wang & F. Na., *UCR2982*, *CBS 140044* (culture); UNITED STATES. CALIFORNIA. Huntington Botanical Gardens, Los Angeles County. Head of *Euwallacea* sp., collected from galleries of infested *Persea americana*, 2013, D.H. Wang & F. Na., *UCR2983*, *CBS 140114* (culture); UNITED STATES. CALIFORNIA. Huntington Botanical Gardens, Los Angeles County. Abdomen of

Euwallacea sp., collected from galleries of infested *Persea americana*, 2013, A. Eskalen, *UCR2984*, *CBS 140115* (culture); UNITED STATES. CALIFORNIA. Pasadena, Los Angeles County. Head of *Euwallacea* sp., collected from galleries of infested *Ricinus communis*, 2013, D.H. Wang, F. Na., & A. Eskalen, *UCR2985*, *CBS 140040* (culture); UNITED STATES. CALIFORNIA. Pasadena, Los Angeles County. Head of *Euwallacea* sp., collected from galleries of infested *Ricinus communis*, 2013, D.H. Wang, F. Na., & A. Eskalen, *UCR2986*, *CBS 140041* (culture); UNITED STATES. CALIFORNIA. Pasadena, Los Angeles County. Head of *Euwallacea* sp., collected from galleries of infested *Ricinus communis*, 2013, D.H. Wang, F. Na., & A. Eskalen, *UCR2987*, *CBS 140116* (culture); UNITED STATES. CALIFORNIA. Huntington Botanical Gardens, Los Angeles County. Head of *Euwallacea* sp., collected from galleries of infested *Acer negundo*, 2013, D.H. Wang & F. Na., *UCR2995*, *CBS 140043* (culture).

Notes: Phylogenetic analyses reveal that *P. pembeum* recovered from the heads of *Euwallacea* sp. #1 collected from avocado, California sycamore, box elder and castor oil plant throughout the Los Angeles Basin is distinct but closely related to *Paracremonium contagium* L. Lombard & Crous, an undescribed species of *Paracremonium* from Vietnam, and *Paracremonium inflatum* L. Lombard & Crous. All three lineages are genetically distinct from *Xenoacremonium recifei*. Conidial dimensions do not differ between lineages. However, conidiophores of *P. pembeum* from California are either simple phialides or groups of 2–3 phialides borne on a stipe, whereas those of the Vietnamese isolates are simple phialides or branches borne on a stipe, giving rise to 1–2 phialides and/or metulae, giving rise to 2–3 phialides. Chlamydospores previously were unseen in *Paracremonium* isolates (Lombard et al. 2015) but were observed in isolates from California. Conidiophores radiating from sterile coils and inconspicuously swollen septa in the hyphae (Lombard et al. 2015) were not observed.

DISCUSSION

Symbioses between ambrosia beetles and mycangial fungi are increasingly seen as dynamic ecosystems with multiple community associates, each with varying fidelity and residency (Kostovcik et al. 2014). In this study six fungal species in two genera are co-occurring mycangial fungal associates of the PSHB based on the presence and incidence of fungal colonies within the heads of adult female beetles. Two of these fungal species, *Graphium euwallaceae* and *Paracremonium pembeum*, are confirmed pathogens of avocado and box elder. The co-occurrence of *Fusarium*, *Graphium* and *"Acremonium"* species had been documented for another ambrosia beetle, *X. ferrugineus* (Baker and Norris 1968), suggesting that these fungi may interact and potentially play a role in the life history of ambrosia beetles. Co-occurrence of *Fusarium* and *Graphium* recently was reported from *E. validus* in Pennsylvania, although results of that study indicate near phoretic exclusivity of *Graphium* fungi on beetles recovered from *Ailanthus altissima* (Kasson et al. 2013). *E. validus*, not unlike the close association between PSHB and *G. euwallaceae*, is associated routinely with a second mycangial fungus, *Raffaelea subfusca*, thus highlighting some interesting parallels between these two fungal-beetle associations. In both systems (i) mycangial community members are present in near equal proportion and (ii) mycangial fusaria appear to be the primary nutritional symbiont for rearing offspring (Kasson et al. 2013). *Graphium* spp. have been reported to be associated with other ambrosia beetle species (Funk 1970, Kolařík and Hulcr 2009) and commonly from bark beetles (Wingfield and Gibbs 1991, Krokene and Solheim 1996, Paciura et al. 2010). This suggests that association with subcortical insects is an important aspect of the ecology of various *Graphium* species but the association does not appear to be as strict as that between the beetle and the primary nutritional fungus.

The genus *Acremonium* had belonged to polyphyletic group ascribed to diverse ascomycete families in many distinct orders (Summerbell et al. 2011). Genera within the Nectriaceae Tul. & C.Tul. (1844) (including *Acremonium*) have been revised based on more comprehensive multilocus phylogenetic analyses (Lombard et al. 2015). *Paracremonium* is a new genus established from a group of fungi previously treated as *Acremonium recifei* (now renamed *Xenoacremonium recifei* L. Lombard & Crous) (Lombard et al. 2015). Until now all species in *Paracremonium* were associated with human infections (Lombard et al. 2015). Of interest *Paracremonium*-like fungi are recovered frequently from mycangia and galleries of many ambrosia beetles. For example, heads of both female and male *E. validus* as well as from non-disinfested heads of both female and male *E. interjectus* and larvae of *E. validus* yielded *Paracremonium*-like colonies (Kasson and Berger unpubl). Similarly fungi preliminarily identified as *Acremonium* are routinely isolated from galleries and surface of ambrosia beetles in the genus *Xylosandrus*, although rarely from mycangia (Bateman and Hulcr unpubl). These observations corroborate the results of this study suggesting that *Paracremonium* or *Acremonium* species are frequent facultative inhabitants of the ambrosia beetle-created environment.

Acremonium was characterized morphologically by septate hyphae giving rise to thin, tapered, mostly lateral phialides produced singly or in small groups, with aseptate, hyaline conidia produced in mucoid heads or unconnected chains (Summerbell et al. 2011). Now conidiophores radiating from sterile coils and inconspicuously swollen septa in the hyphae are attributed as

distinguishing characters of *Paracremonium* from *Xenoacremonium recifei* but are based on a small number of isolates (Lombard et al. 2015). This character was not observed in isolates of *P. pembeum* and *Paracremonium* sp. I (strains from Vietnam), which represent previously undescribed fungal species within *Paracremonium*. The presence of chlamydospores in isolates from California that had gone unseen in *Paracremonium* (Lombard et al. 2015) further suggests that more isolates and taxa may be needed to determine distinguishing characters of this emerging genus. Morphological distinctions of conidia also may be difficult between closely related species given that phylogenetically distant species of "*Acremonium*" have morphologically indistinguishable conidia (Summerbell et al. 2011). Therefore the identical dimensions of conidia in the phylogenetically distinct groups *P. pembeum*, *Paracremonium* sp. I, *P. inflatum*, *P. contagium* and *Xenoacremonium recifei*, may represent a symplesiomorphy.

With 303 tree species prone to attack by the PSHB and 37 tree species suitable for PSHB reproduction, the significance of these fungi for the beetle, such as nutritional and other benefits, may vary substantially between hosts. Differences in colonization, frequency and relative abundance among fungal species in the present study within and between hosts imply that these fungi may play a different role in the life cycle of the PSHB, depending on the host. Similarly in *E. validus* the relative abundance of *Raffaelea subfusca* appears significantly higher from beetles recovered from gymnosperm hosts compared to angiosperm counterparts (MT Kasson, MC Berger unpubl). Apart from castor oil plant *F. euwallaceae* is the most frequently and abundantly recovered fungus in beetle heads collected from different hosts, suggesting that this fungus is the primary food source for the beetle. Further differences in relative abundances of fungi within gallery walls among hosts may suggest different functions of the fungi depending on the host. However, implications may be unclear due to variability in tree age, gallery age and stage of offspring within the gallery at the time of sampling. To understand the role of the fungi for the beetle and host, dietary studies at different developmental stages of the beetle and host dynamics on the 139 host species from which the fungi have been recovered need to be investigated. Dynamics of weevil and rust mutualists have been shown to be largely determined by host-plant dynamics (Bacher and Friedli 2002). Host defensive chemistry ultimately can affect both the ability of ambrosia beetles to successfully attack trees and produce brood and the ability of their associated fungi to grow (Klepzig and Six 2004). With *P. pembeum* growing optimally at warmer temperatures in vitro than *G. euwallaceae* it is possible that temperature and other environmental conditions result in variable abundances of the fungi but also may contribute to stabilization of the multispecies symbiosis, as has been observed with other scolytine beetle associates (Addison et al. 2013). However, at this time nothing is known about whether *G. euwallaceae* or *P. pembeum* may affect the fitness of female PSHB beetles either as a food source or by assisting the beetle in producing brood within different hosts.

Vietnamese isolates recovered from the heads of PSHB comprise a previously undescribed species of *Paracremonium* closely related to *P. pembeum* and comprise three *Graphium* spp., including *G. euwallaceae*, *G. carbonarium* and a previously undescribed species of *Graphium*. In addition, isolates recovered from the heads of the TSHB in Thailand comprise a previously undescribed *Graphium* species. These results indicate that the PSHB is associated with a greater diversity of *Graphium* and *Paracremonium* species in Asia, the putative area of origin of the PSHB. Given that *G. carbonarium* originally was described in association with *Pissodes* sp. on *Salix babylonica* L. in China (Paciura et al. 2010) it is likely that this and related *Graphium* spp. are loosely associated with many subcortical insects in the native region. Lateral transfer of fungal associates within a sympatric community of wood-boring insects has been observed even in nutritional mutualistic fungi and between native and exotic ambrosia beetles (Carrillo et al. 2014). A larger sample size of both beetles and fungi (i.e. populations and loci) throughout southeastern Asia is required to determine the precise mechanisms by which fungal diversity is structured. The diversity of different *Fusarium*, *Graphium* and *Paracremonium* species associated with the PSHB within California may indicate independent introductions of the beetle or a diversity of fungi that arrived with the first beetle population or our limited knowledge of local native fungi in these genera, which may have colonized this invasive ambrosia beetle.

ACKNOWLEDGMENTS

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