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e-**X**tra*

Two Novel Fungal Symbionts *Fusarium kuroshium* sp. nov. and *Graphium kuroshium* sp. nov. of Kuroshio Shot Hole Borer (*Euwallacea* sp. nr. *fornicatus*) Cause Fusarium Dieback on Woody Host Species in California

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Abstract

Shot hole borer (SHB)-Fusarium dieback (FD) is a new pest-disease complex affecting numerous tree species in California and is vectored by two distinct, but related ambrosia beetles (*Euwallacea* sp. nr. *fornicatus*) called polyphagous shot hole borer (PSHB) and Kuroshio shot hole borer (KSHB). These pest-disease complexes cause branch dieback and tree mortality on numerous wildland and landscape tree species, as well as agricultural tree species, primarily avocado. The recent discovery of KSHB in California initiated an investigation of fungal symbionts associated with the KSHB vector. Ten isolates of *Fusarium* sp. and *Graphium* sp., respectively, were recovered from the mycangia of adult KSHB females captured in three different locations within San Diego County and compared with the known symbiotic fungi of PSHB. Multigene phylogenetic analyses of the internal transcribed spacer region (ITS), translation elongation factor-1 alpha (TEF1-α), and RNA polymerase II subunit (RPB1, RPB2) regions as well as morphological comparisons revealed that two novel fungal associates *Fusarium kuroshium* sp. nov. and *Graphium kuroshium* sp. nov. obtained from KSHB were related to, but distinct from the fungal symbionts *F. euwallaceae* and *G. euwallaceae* associated with PSHB in California. Pathogenicity tests on healthy, young avocado plants revealed *F. kuroshium* and *G. kuroshium* to be pathogenic. Lesion lengths from inoculation of *F. kuroshium* were found to be significantly shorter compared with those caused by *F. euwallaceae*, while no difference in symptom severity was detected between *Graphium* spp. associated with KSHB and PSHB. These findings highlight the pest disease complexes of KSHB-FD and PSHB-FD as distinct, but collective threats adversely impacting woody hosts throughout California.

The Scolytinae is a subfamily (Alonso-Zarazaga and Lyal 2009) of bark and wood-boring weevils including more than 6,000 species that are of considerable economic importance in both temperate and tropical forests (Beaver et al. 2014; Smith and Hulcr 2015). Most of these insects typically attack declining or dead trees, but some are economically important species that attack apparently healthy trees in native and invaded areas, such as California. Ambrosia beetles are known to be associated with fungal and bacterial organisms (Beaver et al. 2014; Hulcr et al. 2012), with the fungal symbionts sometimes being phytopathogenic (Eskalen et al. 2013; Hulcr and Dunn 2011; Mendel et al. 2012; Ploetz et al. 2013). Shot hole borer (SHB)-Fusarium dieback (FD) is an invasive pest-disease complex affecting numerous tree species in California. It was first reported on a host in 2012 (Eskalen et al. 2012) and has since been reported to attack over 300 tree species in wildland, landscape, and avocado growing areas (Eskalen et al. 2013; http://eskalenlab.ucr.edu/shotholeborerhosts.html). The extent of economic damage these pests have on native ecosystems in addition to agricultural settings in California is unknown, but the estimated ecological and economic burdens in urban areas imposed by these invasive pests are significant. For example, since 2014, the PSHB-FD complex has infested 1/3 of California sycamore (*Platanus racemosa*) in Orange County public parks and caused the removal of 1,262 trees, resulting in approximately US $4 million in tree removal costs to Orange County parks (OC Parks 2017). Unfortunately, there are no viable management strategies to combat this pest disease complex besides removal of infested branches or trees to reduce beetle populations and hazards to people.

The negative impact ambrosia beetles and their mutualists are having on native and cultivated trees are not only a significant problem in California and other parts of the United States, but are also a significant problem in invaded and native areas throughout the world. For example, the Tea shot hole borer (*Euwallacea fornicatus* Eichoff, "TSHB") associated with *Fusarium ambrosium* is found in India and Sri Lanka (Danthanarayana 1968), where it is a serious pest of tea (*Camellia sinensis*). Invasive ambrosia beetles such as those belonging to the *E. fornicatus* species complex are native to southeast Asia (Beaver 1989; Hulcr and Stelinski 2017; Stouthamer et al. 2017) and have been previously described to form a unique mutualism with *Fusarium* spp. belonging to the Ambrosia Fusaria clade (AFC) (Kasson et al. 2013; O'Donnell et al. 2015). In addition to being associated with AFC members, ambrosia beetles from the *Euwallacea* genus have also been reported to be associated with other fungal genera including *Graphium* spp. (Beaver 1989; Freeman et al. 2016; Kasson et al. 2013; Kirisitis 2007; Lynch et al. 2016), *Paracremonium* spp. (Lynch et al. 2016), and also *Raffaelea subfusca* (Kasson et al. 2013) with *Graphium* spp. proposed to serve as a primary food source for developing larvae, reducing competition with adult females feeding on AFC fusaria (Freeman et al. 2016). *Euwallacea* spp. have been reported to invade multiple areas of the United States including California, Florida, Hawaii, and much of the mid-Atlantic and Southeastern U.S. (CABI 2015; Cognato et al. 2015; Eskalen et al. 2013; O'Donnell et al. 2015; Rabaglia et al. 2006; Short et al. 2017), as well as other parts of the world including Australia, Costa

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Rica, Guatemala, Israel, Panama, and South Africa (CABI 2015; Stouthamer et al. 2017). Invasions of more than a single *Euwallacea* species have occurred in California and Florida (Rabaglia et al. 2006; Stouthamer et al. 2017), with the former invaded by two genetically distinct, but morphologically indistinguishable *Euwallacea* spp. nr. *fornicatus* with independent evolutionary lineages (O'Donnell et al. 2015; Stouthamer et al. 2017), each associated with characteristic symbiotic fungi.

The two *Euwallacea* spp. invading California are now known as the polyphagous shot hole borer (*Euwallacea* sp. nr. *fornicatus* #1., PSHB) (Eskalen et al. 2013; O'Donnell et al. 2015) and Kuroshio shot hole borer (*Euwallacea* sp. nr. *fornicatus* #5, KSHB) (O'Donnell et al. 2015; Stouthamer et al. 2017). PSHB was initially reported on a backyard avocado in Los Angeles County in 2012 (Eskalen et al. 2012) and is known to be associated with three pathogenic fungal symbionts: *F. euwallaceae* S. Freeman, Z. Mendel, T. Aoki & O'Donnell, *Graphium euwallaceae* M. Twizeyimana, S.C. Lynch & A. Eskalen, and *Paracremonium pembeum* S.C. Lynch & Eskalen. Since its initial discovery in 2012, PSHB has spread to several counties throughout southern California, including Orange, Riverside, San Bernardino, and Ventura (http://eskalenlab.ucr.edu/distribution.html). In 2013, new beetle infestations were detected on sycamore trees located in El Cajon in San Diego County. This beetle infestation was initially considered to be an extension of the existing infestation of PSHB in Los Angeles County. Molecular analysis of the fungal symbionts (*Fusarium* spp. and *Graphium* spp.) obtained from KSHB revealed significant differences from the symbionts recovered from PSHB in Los Angeles. The beetles collected from the San Diego infestation were also shown to be distinct from PSHB at the mitochondrial cytochrome oxidase I (COI) locus (Cooperband et al. 2016; Stouthamer et al. 2017). The beetles have since spread to adjacent counties and the two populations are currently sympatric in Orange and southern Los Angeles counties. Since there are currently two invasive SHB-FD complexes in California, it is important to characterize and distinguish the symbiotic fungi associated with the beetle vectors in order to elucidate any differences in pathogenicity, as well as describe morphological and genetic differences for diagnostic detection in affected areas.

In this study, we aimed to characterize the fungal symbionts of KSHB as well as explore the differences between the two groups of fungal symbionts associated with PSHB and KSHB. The primary objectives were to (i) identify morphological characteristics of *Fusarium* spp. and *Graphium* spp. associated with KSHB and resolve differences from other closely related species of the respective symbionts using multigene molecular phylogeny at informative loci, (ii) formally describe the novel fungal pathogens associated with KSHB as *F. kuroshium* and *G. kuroshium*, and (iii) determine the pathogenicity of *F. kuroshium* and *G. kuroshium* on healthy avocado plants and compare it to that of previously described *F. euwallaceae* (Freeman et al. 2013) and *G. euwallaceae* (Lynch et al. 2016) associated with PSHB.

Materials and Methods

Fungal isolate collection. Fungal isolates used in this study were obtained from the heads of beetles, similar to methods described by Lynch et al. (2016), and from wood recovered from KSHB galleries in their reproductive host trees, similar to methods described by Eskalen et al. (2013). A total of 40 beetles and symptomatic wood samples around the beetle galleries were collected from infested avocado (*Persea americana*) and sycamore (*Platanus racemosa*) in four different locations (Bonsall, El Cajon, Escondido, Fallbrook) in San Diego County. The samples were transferred to laboratory in a cooler (CDFA plant pest permit #2887) and processed immediately. The beetles were surface sterilized by submerging in 70% ethanol and vortexed for 20 s, rinsed with sterile deionized water, and allowed to dry on sterile filter paper. Beetle heads were separated from the thoracic and abdominal segments under a dissection microscope, then the segments were macerated in 1.5 ml microcentrifuge tubes with sterile plastic pestles. The macerated heads were suspended in 1 ml of sterile water and 50 µl of the suspensions were pipetted onto Petri plates containing potato dextrose agar (PDA; BD Difco, Sparks, MD) amended with tetracycline hydrochloride (0.01%) and spread using sterile glass rods. Plates were incubated for 5 days at 25°C and fungal colonies were counted and unique morphologies were subcultured for further identification. Five replication plates per beetle head suspension were used to quantify relative fungal content in their mycangia similar to methods described by Lynch et al. (2016). Beetle gallery wall samples were obtained by carving out small pieces of galleries from infested sycamore and avocado wood and sampling of the gallery surfaces was performed by scraping the gallery wall with a sterile wooden toothpick (Kajimura and Hijii 1992). Wooden toothpicks containing the scrapings were rinsed with 1 ml of sterile water in a microcentrifuge tube, and 50 µl of the wash were pipetted onto plates containing PDA amended with tetracycline hydrochloride (0.01%) and spread using sterile glass rods. Plates were incubated at 25°C for 5 days, and individual, single-spore fungal colonies were isolated from the plates.

Fungal DNA extraction, PCR, and phylogenetic analysis. Genomic DNA of the fungal isolates obtained from KSHB beetles and gallery samples was extracted using a modified protocol by Cenis (1992). Briefly, 50 µg of fungal mycelia was harvested from 7-day-old *Fusarium* spp. or 14-day-old *Graphium* spp. culture plates and added to microcentrifuge tubes containing glass beads and 500 µl extraction buffer (200 mM Tris HCL, 250 mM NaCl, 25 mM EDTA, 2% sodium dodecyl sulfate in 250 ml H_2O), then the mixture was processed in a tissue homogenizer (MP Biomedicals, Irvine, CA) to lyse. Sodium acetate (150 µl) was then added to each microcentrifuge tube, and they were placed in a –20°C freezer for 10 min. The microcentrifuge tubes were centrifuged at 14,000 relative centrifugal force (RCF), and then 300 µl of the supernatants were mixed with an equal volume of isopropanol prior to storing at 0°C for 10 min. The precipitated DNA was pelleted by centrifugation at 14,000 RCF, washed with 70% ethanol, air dried, then suspended in TE buffer.

PCR amplification of the internal transcribed spacer (ITS)1-5.8S-ITS2 and translation elongation factor 1-α (TEF1-α) was done using ITS4/ITS5 (White et al. 1990) for all species tested, EF1/EF2 for *Fusarium* spp. only (O'Donnell et al. 1998), and EF1F/EF2R for *Graphium* spp. only (Jacobs et al. 2004). In addition, PCR amplification of RNA polymerase subunit I (RPB1) and RNA polymerase subunit II (RPB2) loci was performed for *Fusarium* spp. only using primers F5/R8 (RPB1-1) (O'Donnell et al. 2010), F7/G2R (RPB1-2) (O'Donnell et al. 2010), 5F2/7CR (RPB2-1) (O'Donnell et al. 2007), and 7CF/11AR (RPB2-2) (O'Donnell et al. 2007). It should be noted that large subunit (LSU) domains D1 and D2 of the LSU rDNA partition were previously found to be least informative (Kasson et al. 2013; O'Donnell et al. 2015), and therefore were not included in this study. Each PCR reaction mixture consisted of 12.5 µl GoTaq DNA polymerase (Promega, Madison, WI), 9.3 µl sterile DNase-free water, 0.6 µl of 10 µM forward primer, 0.6 µl of 10 µM reverse primer PCR, and 2 µl of genomic DNA template, for a total of 25 µl reaction mixture. PCR was performed for each primer set using published cycling parameters (Jacobs et al. 2004; O'Donnell et al. 1998, 2007; White et al. 1990). Amplified products were separated by gel electrophoresis in 1% agarose gel with 0.5× Tris-boric acid-EDTA buffer, stained with SYBR Green (Invitrogen, Carlsbad, CA), and viewed under UV light. Products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA), then sequenced in both directions at the Institute for Integrative Genome Biology, University of California Riverside with corresponding primers used for PCR. Raw sequences were assembled in Sequencher 4.6 (Gene Codes Corp., Ann Arbor, MI).

Multigene phylogenetic analysis was conducted to determine the genetic relatedness of *Fusarium* sp. and *Graphium* sp. isolates obtained from sampled locations where KSHB is present. These isolates were also compared with other members of their respective genera. Phylogenetic analysis of *Fusarium* spp. was conducted using concatenated DNA sequences at ITS, TEF1-α, RPB1, and RPB2 gene regions from 10 isolates of *Fusarium* spp., DNA of 10 isolates of *Fusarium* spp. native to Taiwan (provided by Dr. Chi-Yu Chen) (Table 1), along with DNA sequences obtained from GenBank (Table 2) from 26 isolates previously used in AFC phylogenetic analysis from Kasson et al. (2013) and O'Donnell et al. (2015). Phylogenetic analysis of

Graphium spp. was conducted using concatenated DNA sequences of ITS and TEF1-α gene regions from 10 isolates of *Graphium* spp. (Table 1) as well as 36 isolates obtained from GenBank (Table 2) used in previous analysis done by Lynch et al. (2016). All respective sequences from *Fusarium* spp. and *Graphium* spp. analysis used were aligned using Clustal X (Thompson et al. 1997) and concatenated after alignment. A partition file was created to indicate the range of each gene in the concatenated alignment and allow for different substitution models. The multigene phylogenies were constructed with maximum likelihood (ML) methods RAxML version 8.2.8 (Stamatakis 2014) and IQ-TREE (Nguyen et al. 2015). PartitionFinder was used to determine that the best partitioning scheme was EF, ITS, and RPB1+RPB2. RAxML was run with the rapid bootstrap approach, generating 1,000 bootstrap replicates, followed by a thorough ML search. IQ-TREE was run with 1,000 standard bootstrap analyses to generate final tree run. The ModelFinder option identified the best substitution model for each of the three defined partitions (-m MFP -nt AUTO -spp partitions.txt -b 1000) for *Fusarium* spp. and for each of the two defined partitions (-bb 1000 -alrt 1000 -m TESTNEW -q partitions.txt) for *Graphium* spp. tested.

Morphological characterization. Morphological characteristics of *Fusarium* spp. and *Graphium* spp. were observed using a compound microscope (Leica DMLB, Wetzlar, Germany) with a SPOT camera attachment (Diagnostic Instruments, Sterling Heights, MI) using 10 isolates of each fungal species. *Fusarium* spp. cultures were grown on PDA and synthetic low-nutrient agar (SNA; Nirenberg 1976) in complete darkness, under near UV light (F40T12/BLB 40W, Philips, Amsterdam, Netherlands), and under constant light (F40T12/CW 40W, Philips, Amsterdam, Netherlands), at ambient room temperature (23 to 25°C), and examined at 14 days and 1 month. *Graphium* spp. cultures were grown on PDA and oat meal agar (OMA) (Gams et al. 1998) under constant light (F40T12/CW 40W, Philips, Amsterdam, Netherlands) at ambient room temperature and examined at 14 days. Cultures of *Fusarium* spp. on PDA in 6-cm Petri dishes at 25°C in darkness were used to characterize colony color and morphology. Fourteen-day-old cultures of *Fusarium* spp. were used to evaluate microscopic characters on SNA and PDA similar to Aoki et al. (2005) and Freeman et al. (2013). The Munsell (1976) color palette was used as the color standard. Cultures of *Graphium* spp. were grown on PDA in 6-cm Petri dishes at 25°C to evaluate colony color, morphology, and microscopic characters. The lengths and widths of the *Fusarium* spp. and *Graphium* spp. conidia were measured from cultures using SPOT imaging software (Diagnostic Instruments, Sterling Heights, MI).

Growth rates of three isolates of each *Fusarium* spp. and *Graphium* spp., respectively, were obtained by measuring the fungal colony diameters of cultures incubated at various temperatures between 5 and 40°C (5°C increments) in replicates of three. Plugs of agar containing the fungi were obtained from the edges of 1-week-old cultures and placed on Petri plates containing PDA. Diameters of the *Fusarium* spp. colonies were measured every day for 9 days, and *Graphium* spp. were measured every 2 days for 14 days. This experiment was repeated once.

Pathogenicity assay. Pathogenicity assays were conducted under greenhouse conditions, in which 2-year-old avocado cv. Zutano seedlings were each inoculated with *F. euwallaceae*, *Fusarium* spp., *G. euwallaceae*, or *Graphium* spp. using similar methods described by Twizeyimana et al. (2013). Two isolates from each fungal species were used in the pathogenicity assay, and each treatment was assigned by complete randomized design with 10 replicates across two trials. Ten avocado plants were used per fungal species and control in each

Table 1. Representative isolates of *Fusarium* spp. and *Graphium* spp. from *Euwallacea* spp. and various hosts obtained in this study

Isolate	Species	Host	Location[a]	GenBank numbers[b]			
				ITS	EF1-α	RPB1	RPB2
UCR3641[cd]	*Fusarium* sp.	*Platanus racemosa*	El Cajon, CA	KX262196	KX262216	KX262236	KX262256
UCR3644[d]	*Fusarium* sp.	*P. racemosa*	El Cajon, CA	KX262197	KX262217	KX262237	KX262257
UCR3651	*Fusarium* sp.	*Euwallacea* sp.	Fallbrook, CA	KX262198	KX262218	KX262238	KX262258
UCR3653	*Fusarium* sp.	*Persea americana*	Bonsall, CA	KX262200	KX262220	KX262240	KX262260
UCR3654	*Fusarium* sp.	*P. americana*	Bonsall, CA	KX262201	KX262221	KX262241	KX262261
UCR3657	*Fusarium* sp.	*Euwallacea* sp.	Bonsall, CA	KX262202	KX262222	KX262242	KX262262
UCR3659	*Fusarium* sp.	*Euwallacea* sp.	Bonsall, CA	KX262203	KX262223	KX262243	KX262263
UCR3660	*Fusarium* sp.	*Euwallacea* sp.	Bonsall, CA	KX262204	KX262224	KX262244	KX262264
UCR3661	*Fusarium* sp.	*P. americana*	Escondido, CA	KX262205	KX262225	KX262245	KX262265
UCR3662	*Fusarium* sp.	*P. americana*	Escondido, CA	KX262199	KX262219	KX262239	KX262259
UCR4672[e]	*Fusarium* sp.	*Euwallacea* sp.	Taichung, Taiwan	KX262206	KX262226	KX262246	KX262266
UCR4673[e]	*Fusarium* sp.	*Euwallacea* sp.	Taichung, Taiwan	KX262207	KX262227	KX262247	KX262267
UCR4674[e]	*Fusarium* sp.	*Euwallacea* sp.	Taichung, Taiwan	KX262208	KX262228	KX262248	KX262268
UCR4675[e]	*Fusarium* sp.	*Euwallacea* sp.	Taichung, Taiwan	KX262209	KX262229	KX262249	KX262269
UCR4676[e]	*Fusarium* sp.	*Euwallacea* sp.	Taichung, Taiwan	KX262210	KX262230	KX262250	KX262270
UCR4677[e]	*Fusarium* sp.	*Euwallacea* sp.	Taichung, Taiwan	KX262211	KX262231	KX262251	KX262271
UCR4678[e]	*Fusarium* sp.	*Euwallacea* sp.	Taichung, Taiwan	KX262212	KX262232	KX262252	KX262272
UCR4679[e]	*Fusarium* sp.	*Euwallacea* sp.	Taichung, Taiwan	KX262213	KX262233	KX262253	KX262273
UCR4680[e]	*Fusarium* sp.	*Euwallacea* sp.	Taichung, Taiwan	KX262214	KX262234	KX262254	KX262274
UCR4681[e]	*Fusarium* sp.	*Euwallacea* sp.	Taichung, Taiwan	KX262215	KX262235	KX262255	KX262275
UCR4593[cd]	*Graphium* sp.	*P. americana*	Fallbrook, CA	KX262276	KX262286	...	...
UCR4594[d]	*Graphium* sp.	*P. americana*	Fallbrook, CA	KX262277	KX262287	...	...
UCR4606	*Graphium* sp.	*P. americana*	Bonsall, CA	KX262278	KX262288	...	...
UCR4607	*Graphium* sp.	*P. americana*	Bonsall, CA	KX262279	KX262289	...	...
UCR4608	*Graphium* sp.	*Euwallacea* sp.	Bonsall, CA	KX262280	KX262290	...	...
UCR4609	*Graphium* sp.	*Euwallacea* sp.	Bonsall, CA	KX262281	KX262291	...	...
UCR4616	*Graphium* sp.	*P. americana*	Escondido, CA	KX262282	KX262292	...	...
UCR4617	*Graphium* sp.	*P. americana*	Escondido, CA	KX262283	KX262293	...	...
UCR4618	*Graphium* sp.	*Euwallacea* sp.	Escondido, CA	KX262284	KX262294	...	...
UCR4622	*Graphium* sp.	*Euwallacea* sp.	Escondido, CA	KX262285	KX262295	...	...

[a] Taiwanese fungal isolates collected and sequenced by C. Y. Chen.
[b] ITS: internal transcribed spacer regions; EF1-α: translation elongation factor 1-α; RPB: RNA II polymerase subunit.
[c] Denotes type-specimen.
[d] Isolates used in pathogenicity test.
[e] DNA only.

Table 2. Descriptions and sequences of fungi obtained from GenBank used in the phylogenetic analysis

Isolate	Species	Host	Origin	GenBank numbers[a]			
				ITS	EF1-α	RPB1	RPB2[b]
NRRL20438	*Fusarium ambrosium* [AF-1]	*Euwallacea fornicatus*	India	AF178397	AF178332	JX171470	JX171584
NRRL22231	*Fusarium* sp. [AF-5]	*Hevea brasiliensis*	Malaysia	KC691570	KC691542	KC691600	KC691631, KC691660
NRRL22346	*F. ambrosium* [AF-1]	*E. fornicatus*	India	EU329669	FJ240350	KC691587	EU329503
NRRL22468	*F. neocosmosporiellum*	*Arachis hypogaea*	Guinea	DQ094318	AF178349	KC691616	EU329512
NRRL22643	*Fusarium* sp. [AF-9]	*Xyleborus ferrigineus*	Costa Rica	KC691583	DQ247628	KC691613	KC691644, KC691673
NRRL32434	*F. lichenicola*	*Homo sapiens*	Germany	DQ094444	DQ246977	HM347156	EF470161
NRRL43467	*F. neocosmosporiellum*	*Homo sapiens*	LA (U.S.A.)	EF453092	EF452940	HM347178	EF469979
NRRL46518	*Fusarium* sp. [AF-5]	*H. brasiliensis*	Malaysia	KC691571	KC691543	KC691601	KC691632, KC691661
NRRL54722	*Fusarium* sp. [AF-2]	*Euwallacea* sp.	Israel	JQ038014	JQ038007	JQ038021	JQ038028
NRRL54723	*Fusarium* sp. [AF-2]	*Euwallacea* sp.	Israel	JQ038015	JQ038008	JQ038022	JQ038029
NRRL54724	*Fusarium* sp. [AF-2]	*Euwallacea* sp.	Israel	JQ038016	JQ038009	JQ038023	JQ038030
NRRL54725	*Fusarium* sp. [AF-2]	*Euwallacea* sp.	Israel	JQ038017	JQ038010	JQ038024	JQ038031
NRRL54726	*Fusarium* sp. [AF-2]	*Euwallacea* sp.	Israel	JQ038018	JQ038011	JQ038025	JQ038032
NRRL54727	*Fusarium* sp. [AF-2]	*Euwallacea* sp.	Israel	JQ038019	JQ038012	JQ038026	JQ038033
NRRL62578	*Fusarium* sp. [AF-4]	*E. validus*	PA (U.S.A.)	KC691565	KC691537	KC691595	KC691626, KC691655
NRRL62579	*Fusarium* sp. [AF-4]	*E. validus*	PA (U.S.A.)	KC691566	KC691538	KC691596	KC691627, KC691656
NRRL62584	*Fusarium* sp. [AF-8]	*Euwallacea* sp.	FL (U.S.A.)	KC691582	KC691554	KC691612	KC691643, KC691672
NRRL62585	*Fusarium* sp. [AF-8]	*Euwallacea* sp.	FL (U.S.A.)	KC691577	KC691549	KC691607	KC691638, KC691667
NRRL62590	*Fusarium* sp. [AF-6]	*Euwallacea* sp.	FL (U.S.A.)	KC691574	KC691546	KC691604	KC691635, KC691664
NRRL62591	*Fusarium* sp. [AF-6]	*Euwallacea* sp.	FL (U.S.A.)	KC691573	KC691545	KC691603	KC691634, KC691663
NRRL62606	*Fusarium* sp. [AF-3]	*Euwallacea* sp.	FL (U.S.A.)	KC691561	KC691533	KC691591	KC691622, KC691651
NRRL62610	*Fusarium* sp. [AF-7]	*Euwallacea* sp.	Australia	KC691575	KC691547	KC691605	KC691636, KC691665
NRRL62611	*Fusarium* sp. [AF-7]	*Euwallacea* sp.	Australia	KC691576	KC691548	KC691606	KC691637, KC691666
NRRL62629	*Fusarium* sp. [AF-3]	*E. interjectus*	FL (U.S.A.)	KC691564	KC691536	KC691594	KC691625, KC691654
NRRL62941	*Fusarium* sp. [AF-10]	unknown	Singapore	KM406633	KM406626	KM406640	KM406647
NRRL62942	*F. ambrosium*	*Camellia sinensis*	Sri Lanka	KM406631	KM406624	KM406638	KM406645
NRRL62944	*Fusarium* sp. [AF-11]	*C. sinensis*	Sri Lanka	KM406634	KM406627	KM406641	KM406648
NRRL66088	*Fusarium* sp.	*Delonix regia*	FL (U.S.A.)	KM406632	KM406632	KM406639	KM406646
UCR2974	*Graphium euwallaceae*	*Ricinus communis*	CA (U.S.A.)	KF540218	KF534799	...	...
UCR2975	*G. euwallaceae*	*Acer negundo*	CA (U.S.A.)	KF540219	KF534800	...	...
UCR2976	*G. euwallaceae*	*R. communis*	CA (U.S.A.)	KF540220	KF534801	...	...
UCR2977	*G. euwallaceae*	*Acacia floribunda*	CA (U.S.A.)	KF540221	KF534802	...	...
UCR2978	*G. euwallaceae*	*Erythrina atitlanensis*	CA (U.S.A.)	KF540222	KF534803	...	...
UCR2979	*G. euwallaceae*	*Quercus agrifolia*	CA (U.S.A.)	KF540223	KF534804	...	...
UCR2980[c]	*G. euwallaceae*	*Persea americana*	CA (U.S.A.)	KF540224	KF534805	...	...
UCR2981	*G. euwallaceae*	*P. americana*	CA (U.S.A.)	KF540225	KF534806	...	...
UCR2308	*G. euwallaceae*	*Acacia auriculiformis*	Vietnam	KM592371	KM592363	...	...
UCR2159	*Graphium* sp. I	*Ailanthus altissima*	PA (U.S.A.)	KJ131228	KJ131238	...	...
UCR2160	*Graphium* sp. I	*A. altissima*	PA (U.S.A.)	KJ131229	KJ131239	...	...
UCR2162	*Graphium* sp. I	*A. altissima*	PA (U.S.A.)	KJ131231	KJ131241	...	...
UCR2163	*Graphium* sp. I	*A. altissima*	PA (U.S.A.)	KJ131232	KJ131242	...	...
UCR2164	*Graphium* sp. I	*A. altissima*	PA (U.S.A.)	KJ131233	KJ131243	...	...
UCR2165	*Graphium* sp. I	*A. altissima*	PA (U.S.A.)	KJ131234	KJ131244	...	...
UCR2166	*Graphium* sp. I	*A. altissima*	PA (U.S.A.)	KJ131235	KJ131245	...	...
UCR2132	*Graphium* sp. II	*Durio* sp.	Thailand	KM592367	KM363259	...	...
UCR2137	*Graphium* sp. II	*Durio* sp.	Thailand	KJ131236	KJ131246	...	...
UCR2140	*Graphium* sp. II	*Durio* sp.	Thailand	KJ131237	KJ131247	...	...
UCR2289	*Graphium* sp. III	*A. auriculiformis*	Vietnam	KM592368	KM592360	...	...
UCR2291	*Graphium* sp. III	*A. auriculiformis*	Vietnam	KM592369	KM592361	...	...
UCR2300	*G. carbonarium*	*A. auriculiformis*	Vietnam	KM592370	KM592362	...	...
UCR2325	*G. carbonarium*	*R. communis*	Vietnam	KM592372	KM592364	...	...
UCR2329	*G. carbonarium*	*R. communis*	Vietnam	KM592373	KM592365	...	...
CMW12418	*G. carbonarium*	*Salix babylonica*	China	FJ434980	HM630602	...	...
CMW12420[c]	*G. carbonarium*	*S. babylonica*	China Salomon	FJ434989	HM630603	...	...
JCM 9300	*G. basitruncatum*	Forest soil	Islands	AB038427	KJ131248	...	...
CMW30626[c]	*G. fabiforme*	*Adansonia rubrostipa*	Madagascar	GQ200616	HM630592	...	...
CMW30627	*G. fabiforme*	*A. rubrostipa*	Madagascar	GQ200617	HM630593	...	...
CMW5605[c]	*G. fimbriisporum*	*Picea abies*	France	AY148177	HM630590	...	...
CMW5606	*G. fimbriisporum*	*P. abies*	Austria	AY148180	HM630591	...	...
CMW5601[c]	*G. laricis*	*Larix decidua*	Austria	AY148183	HM630588	...	...
CMW5603	*G. laricis*	*L. decidua*	Austria	AY148182	HM630589	...	...
CMW5292	*G. penicillioides*	*Populus nigra*	Czech Republic	HQ335310	HM630600	...	...
CMW5295	*G. penicillioides*	*P. nigra*	Czech Republic	HQ335311	HM630601	...	...
CMW503[c]	*G. pseudormiticum*	*Pinus* sp.	South Africa	AY148186	HM630586	...	...
CMW12285	*G. pseudormiticum*	*Tsuga dumosa*	China	HM630608	HM630587	...	...

[a] ITS = internal transcribed spacer region; EF1-α = translation elongation factor 1-α; RPB = DNA-directed RNA polymerase II subunit.
[b] Two accession numbers correspond to unjoined RPB2-1 and RPB2-2 sequences.
[c] Denotes type-specimens.

trial. The fungal cultures used during this experiment were grown on PDA for 7 days (*F. euwallaceae*, *Fusarium* sp.) or 14 days (*G. euwallaceae*, *Graphium* sp.) at 25°C, and agar plugs 5 mm in diameter were cut from the cultures with a sterile cork borer. Upon inoculation, the 5-mm-diameter cork borer was flame-sterilized and used to create wounds in the avocado stems by boring into the xylem to allow pathogens to colonize the wood tissue. The wounds were filled with the agar plugs containing the fungal mycelium side down, then wrapped with Parafilm coated with petroleum jelly. Control plants were wounded in the same manner with a cork borer and sterile agar plugs were applied to the wounds. The inoculated plants were grown for 30 days in the greenhouse prior to destructive sampling.

The wood tissues of the inoculated plants were examined for visible lesions (necrotic tissue) originating from points of fungal inoculation and lesion lengths were measured from the edges of visible discoloration. Wood tissue from the xylem was collected from the margins of lesions, embedded on PDA plates amended with tetracycline hydrochloride (0.01%), and incubated at 25°C for 5 days to recover the fungi from the inoculated plants. Presence of *Fusarium* or *Graphium* in each xylem sample was verified by culturing and subsequent identification sequencing the TEF1-α gene to fulfill Koch's postulates.

Statistical analyses. All statistical analyses were performed using R (version 3.2.3). Analysis of microscopic characters were performed by analysis of variance (ANOVA) using the CAR package (Fox et al. 2016) and means, standard deviation, standard error, max, and min values were calculated implementing the PLYR package (Wickham 2011). Xylem lesion lengths were analyzed by using regression in the LME4 package (Bates et al. 2015) to model the response variable of lesion length by independent factors: fungal species (*F. euwallaceae*, *Fusarium* sp., *G. euwallaceae*, and *Graphium* sp.) and trial. Data were transformed via $\log_{10}$ data transformation but the assumption of normality and homogeneity of variance were both violated. Therefore, a post hoc Tukey's bootstrap test was performed at $\alpha = 0.05$ with specified contrast to control groups. All plots were made using Sigma Plot (version 11; Systat Software Inc., Point Richmond, CA).

Results

Phylogenetic analysis. Sequences of *F. neocosmosporiellum* (NRRL 22468, NRRL 43467) and *F. lichenicola* (NRRL 32434) were used as outgroups for rooting the *Fusarium* trees based on prior analyses of AFC members (Kasson et al. 2013; O'Donnell et al. 2015). Multilocus phylogenetic analysis performed on four loci (ITS, TEF1-α, RPB1, and RPB2) from *Fusarium* isolates tested in this study indicate *Fusarium* sp. (AF-12) recovered from KSHB as a distinct phylogenetic species from *F. euwallaceae* (AF-2) recovered from PSHB and other closely related *Fusarium* spp. (minimum 91% bootstrap support) (Fig. 1, Supplementary Fig. S1). Statistical confidence in the phylogenetic clades was assessed by comparing bootstrap support across multiple analysis methods (maximum likelihood, maximum parsimony [data not shown], neighbor-joining [data not shown]). The recovered tree topologies found strong support for reciprocal monophyly of the *F. euwallaceae* (AF-2) strains (NRRL 54722-54726) and *Fusarium* sp. (AF-12) strains (UCR 3641, 3644, 3651-3654, 3657-3661) analyzed. All *Fusarium* sp. (AF-12) strains and *Fusarium* spp. strains from Taiwan (UCR 4672-4681) reside in AFC major clade B (Kasson et al. 2013; O'Donnell et al. 2015) containing AF-[1-7;10-12] strains; no isolates examined in this study were found to be members of AFC clade A containing AF-[8-9] strains. Strains recovered from *Euwallacea* spp. originating from Taiwan are paraphyletic, with the AF-[13-15] strains showing closer relation to *F. euwallaceae* (AF-2), *Fusarium* sp. (AF-12), and *Fusarium* spp. (AF-[3,4]) compared with AF-16 strains, which are sister lineages to this resolved clade.

Four phylogenetic species (AF-[13-16]) recovered from beetles in Taiwan can be identified from our analysis and were found to be largely divided into four groups. *Fusarium* sp. AF-13 (UCR 4674, 4675) is sister to *Fusarium* sp. (AF-12). *Fusarium* sp. AF-14 Taiwan (UCR 4672, 4681) is part of an unresolved clade and equidistant to the closest relatives *F. euwallaceae* (AF-2), *Fusarium* spp. (AF-12), and *Fusarium* sp. AF-3 FL (U.S.A.) (NRRL 62606, 62629). *Fusarium* sp. AF-15 Taiwan (UCR4679) is sister to *Fusarium* sp. AF-4 Pennsylvania (U.S.A.) (NRRL 62578, 62579). *Fusarium* sp. AF-16 Taiwan (UCR4680, 4673, 4678, 4676, 4677) forms a distinct monophyletic clade that is sister to the clade containing the prior described lineages of AF-[2-4;12-15].

Sequences from previous phylogenetic analysis of *Graphium* spp. (Lynch et al. 2016) were used to build an unrooted phylogenetic tree (Fig. 2, Supplementary Fig. S2). *Graphium* sp. recovered from KSHB in California were found to be genetically distinct from *G. euwallaceae* recovered from PSHB California through multilocus phylogenetic analysis using two informative loci, ITS and TEF1-α. The phylogenetic analysis resolved all *Graphium* sp. isolates associated with

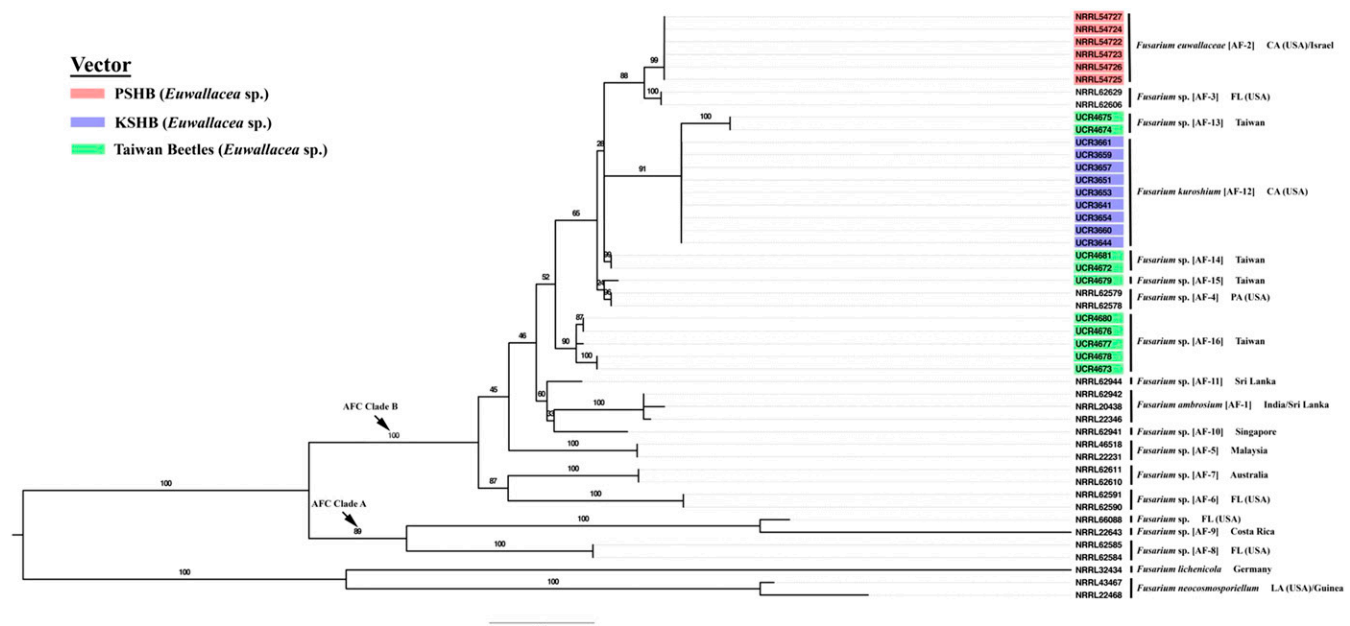

Fig. 1. Multilocus phylogenetic analysis of Ambrosia Fusaria clade (AFC) fusaria conducted with four genes: ribosomal internal transcribed spacer (ITS), elongation factor 1-α (EF1-α), DNA-directed RNA polymerase II largest subunit (RPB1), and DNA-directed RNA polymerase II second largest subunit (RPB2). Diagram was constructed using IQ-TREE maximum likelihood method bootstrapped with 1,000 replications. The highlighted boxes indicate the beetle vector the fungi were recovered from.

KSHB in a monophyletic clade (83% bootstrap support) that was distinct from a well-supported clade of *G. euwallaceae* isolates (94% bootstrap support). Statistical confidence in the phylogenetic clades was assessed by comparing bootstrap support across multiple analysis methods (maximum likelihood, maximum parsimony [data not shown], and neighbor-joining [data not shown]). The recovered tree topologies found strong support for reciprocal monophyly of the *G. euwallaceae* strains (UCR 2308, 2974-2981) and *Graphium* sp. strains (UCR 4593, 4594, 4606-4609, 4616-4618, 4622) analyzed. Isolates associated with wood-boring beetles form a well-supported clade that contained *G. euwallaceae*, *G. carbonarium*, *Graphium* spp. (I-III) (Lynch et al. 2016), and *Graphium* sp. (KSHB). *G. basitruncatum*, previously reported in galleries of the ambrosia beetle *Megaplatypus mutatus* (Ceriani-Nakamurakare et al. 2016), was found to be sister to the clade containing *G. kuroshium* and *G.euwallaceae*. *G. penicilliodes*, *G. fabiforme*, *G. fimbriisporum*, *G. pseudormiticum*, and *G. laricis* were are all sister to the clade containing *Graphium* sp. (KSHB) and *G. euwallaceae*.

Fusarium kuroshium **F. Na, J. D. Carrillo & A. Eskalen, sp. nov.** (Figures 1, 3, 5, and 8). MycoBank MB 821907.

Typification. United States, California. El Cajon, San Diego County. Surface of *Euwallacea* sp. galleries in infested California sycamore (*Platanus racemosa*), 14 December 2013, A. Eskalen (holotype BPI910340 = UCR3641).

Etymology. Derived from the common name of the ambrosia beetle vector, Kuroshio Shot Hole Borer (Stouthamer et al. 2017)

No teleomorph observed. *F. kuroshium* grown on PDA in the dark has average radial mycelial growth rates of 3.5 mm/day at 20°C and 6.5 mm/day at 25°C (Fig. 5). Colonies are initially white (A1) to cream (4A2) in color, and change to grayish red (10D4) with white aerial mycelium in 14 days. Color darkens to grayish ruby (12E5) with age. Reverse pigmentation of colonies grown on PDA in the dark were reddish brown (9D7) and reverse pigmentation becomes reddish brown (9E7) with age. Sporodochial pigmentation starts as light orange (5A4), becoming bluish green (25B8) to grayish green (25D7) with age. Cultures grown under continuous light are initially white (A1) to cream (4A2) in color, and change to brownish orange (5C4). Reverse colony pigmentation is also brownish orange (5C4) with a bluish gray (23D3) ring. Sporodochial pigmentation under continuous light starts reddish yellow (4A6) later becoming grayish green (25E7). Cultures grown on SNA formed colonies with no pigmentation and produced abundant aerial mycelium. Microconidia and macroconidia are produced on long aerial conidiophores with thin walls that are monophialidic and occasionally branched in conidiophores bearing macroconidia. Chlamydospores form in large numbers within hyphae and were also found in mature macroconidia. They are globose to ellipsoidal and can be intercalary or terminal in chains or singular with mostly a pale color and smooth, but can become pigmented with a rough texture containing bluish to brownish hues with time, 3.5 to 10.5 × 3.0 to 9.0 µm total range. No sclerotia are present. Septation within macroconidia occurs over time as the conidia mature; up to five septa per conidium were observed in 14-day-old cultures. Aerial microconidia mostly oval, occasionally reniform or fusiform, with 0 to 1 (–2) septation, zero-septate on SNA: 4.0 to 10.5 × 2.5 to 4.0 µm total range, 6.4 ± 1.4 × 3.1 ± 0.3 µm on average (ex type: 4.5 to 7.5 × 3 to 4.0 µm total range, 6.3 ± 0.9 × 3.3 ± 0.4 µm on average); two-celled oval one-septate on SNA: 5.5 to 13.0 × 3.5 to 5.0 µm total range, 11.4 ± 1.3 × 4.0 ± 0.70 µm (ex type: 6.0 to 12.5 × 3.0 to 4.5 µm total range, 9.3 ± 1.8 × 3.7 ± 0.5 µm on average). Macroconidia are sparsely distributed along with microconidia in culture but are found in abundance within sporodochial structures where they are clustered on thin walled conidiophores that are monophialidic. Macroconidia shape is relatively wide with the dorsal side more curved than the ventral side, wider toward the basal cells, which are barely notched. The apical cell morphology is papillate and round, 3 to 4 (–5) septate formed on PDA and SNA after 14 days. Three septate on SNA in the dark: 15.0 to 20.5 × 3.0 to 7.5 µm total range, 18.1 ± 1.0 × 5.6 ± 0.6 µm on average (ex type: 16.0 to 20.0 × 5.0 to 6.5 µm total range, 18.40 ± 0.8 × 5.7 ± 0.5 µm on average), on SNA under NUV light: 17.0 to 28.5 × 5.5 to 8.0 µm total range, 24.7 ± 1.8 × 6.6 ± 0.5 µm on average (ex type: 20.0 to 27.0 × 5.5 to 8.0 µm total range, 24.9 ± 1.8 × 6.6 ± 0.7 µm on average), on PDA in the dark: 16.0 to 20.5 × 4.0 to 6.5 µm total range, 17.9 ± 0.9 × 5.3 ± 0.5 µm on average (ex type: 16.5 to 19.5 × 4.5 to 6.0 µm total range, 17.7 ± 0.8 × 5.4 ± 0.5 µm on average); four septate on SNA in the dark: 17.5 to 24.0 × 4.5 to 7.5 µm total range, 20.0 ± 1.2 × 5.8 ± 0.5 µm on average (ex type: 18.5 to 22.5 × 5.0 to 7.0 µm total range, 20.2 ± 1.2 × 6.1 ± 0.5 µm on average), on SNA under NUV light: 22.5 to 32.0 × 5.0 to 9.0 µm total range, 27.6 ± 2.0 × 6.8 ± 0.7 µm on average (ex type: 23.5 to 29.5 × 6.0 to 8.0 µm total range, 26.5 ± 1.5 × 6.6 ± 0.5 µm on average), on PDA in the dark: 17.0 to 24.0 × 4.5 to 7.0 µm total range, 19.4 ± 1.3 × 5.6 ± 0.6 µm on average (ex type: 18.0 to 21.0 × 4.5 to 6.5 µm total range, 19.3 ± 0.9 × 5.5 ± 0.6 µm on average).

Known host range. Acer negundo, *Albizia julibrissin*, *Baccharis salicifolia*, *Baccharis pilularis*, *Dombeya cacuminum*, *Erythrina humeana*, *Persea americana*, *Populus fremontii*, *Populus nigra*, *Platanus racemosa*, *Quercus agrifolia*, *Quercus suber*, *Ricinus*

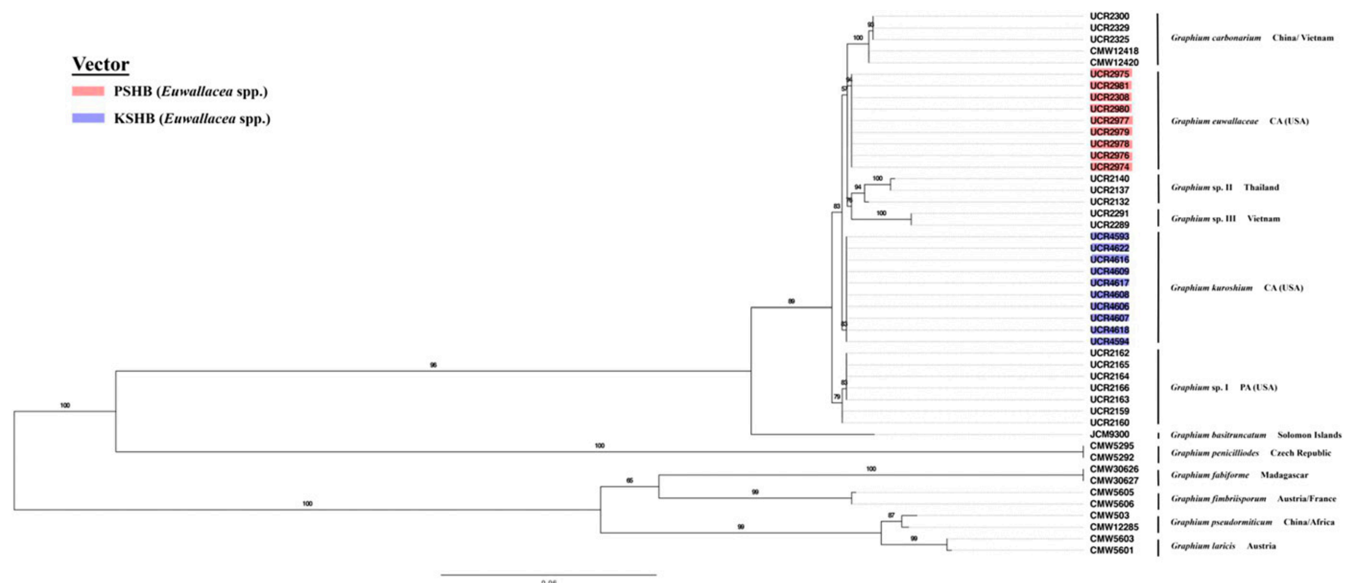

Fig. 2. Multilocus phylogenetic analysis of *Graphium* spp. conducted with two genes: ribosomal internal transcribed spacer (ITS) and elongation factor 1-α (EF1-α). Diagram was constructed using IQ-TREE maximum likelihood method bootstrapped with 1,000 replications. The highlighted boxes indicate the beetle vector the fungi were recovered from.

communis, *Robinia pseudoacacia*, *Salix gooddingii*, *Salix laevigata*, *Salix lasiolepis*, *Tamarix ramosissima*.

Ex-type culture. CBS142642 = Francis Na UCR3641

Fungal distribution. San Diego County, Orange County, California, U.S.A.

Additional specimens examined. United States, California. El Cajon, San Diego County. Surface of *Euwallacea* sp. galleries in *P. racemosa*, 2014, F. Na, UCR3644. United States, California. Fallbrook, San Diego County. Head of *Euwallacea* sp. in infested *Persea americana*, 2015, F. Na, UCR3651. United States, California. Bonsall, San Diego County. Surface of *Euwallacea* sp. galleries in *P. americana*, 2015, F. Na, UCR3653. United States, California. Bonsall, San Diego County. Surface of *Euwallacea* sp. galleries in *P. americana*, 2015, F. Na, UCR3654. United States, California. Bonsall, San Diego County. Head of *Euwallacea* sp. in infested *P. americana*, 2015, F. Na, UCR3653. United States, California. Bonsall, San Diego County. Head of *Euwallacea* sp. in infested *P. americana*, 2015, F. Na, UCR3657. United States, California. Bonsall, San Diego County. Head of *Euwallacea* sp. in infested *P. americana*, 2015, F. Na, UCR3659. United States, California. Bonsall, San Diego County. Head of *Euwallacea* sp. in infested *P. americana*, 2015, F. Na, UCR3660. United States, California. Escondido, San Diego County. Surface of *Euwallacea* sp. galleries in *P. americana*, 2015, F. Na, UCR3661. United States, California. Escondido, San Diego County. Surface of *Euwallacea* sp. galleries in *P. americana*, 2015, F. Na, UCR3662.

Comments. F. kuroshium resembles the related ambrosia fusaria clade (O'Donnell et al. 2015) member *F. euwallaceae* in various morphological features. *F. kuroshium* has been observed to differ from *F. euwallaceae* in colony pigmentation, which is lighter in color on PDA. Phylogenetic analysis shows that *F. kuroshium* is genetically distinct from *F. euwallaceae*, and that it is more closely related to several species of ambrosial *Fusarium* species obtained from *Euwallacea* sp. in Taichung, Taiwan, compared with *F. euwallaceae*.

***Graphium kuroshium* F. Na, J. D. Carrillo & A. Eskalen, sp. nov.** (Figures 2, 4, 6, and 8). MycoBank MP 821908.

Typification. United States, California. Fallbrook, San Diego County. Surface of *Euwallacea* sp. galleries in infested avocado (*Persea americana*), 20 November 2015, A. Eskalen (holotype BPI910341).

Etymology. Derived from the common name of the vector ambrosia beetle, Kuroshio Shot Hole Borer (Stouthamer et al. 2017)

G. kuroshium grown on PDA in the dark has radial mycelial growth rates on average of 1.3 mm/day at 20°C and 3.1 mm/day at 25°C (Fig. 6). Colony growth is initially white (1A), and colony changes in color to olive (3E4-3F3) with yellow green white margins (3B2) in 14-day-old cultures. Reverse pigmentation was olive (3D4) to a darker olive (3D4) in older cultures. Aerial mycelium on PDA was septate and hyaline. Colonies on OMA in the dark showed similar coloration to PDA with sparse mycelial growth with a rough surface. Colonies on PDA under continuous light are initially white (1A) and become olivaceous gray (3B2) with slate gray edges (3B2). Reverse pigmentation is stone gray (3E2). Colonies on OMA under continuous light are initially white (1A) and become olive (3E3) with

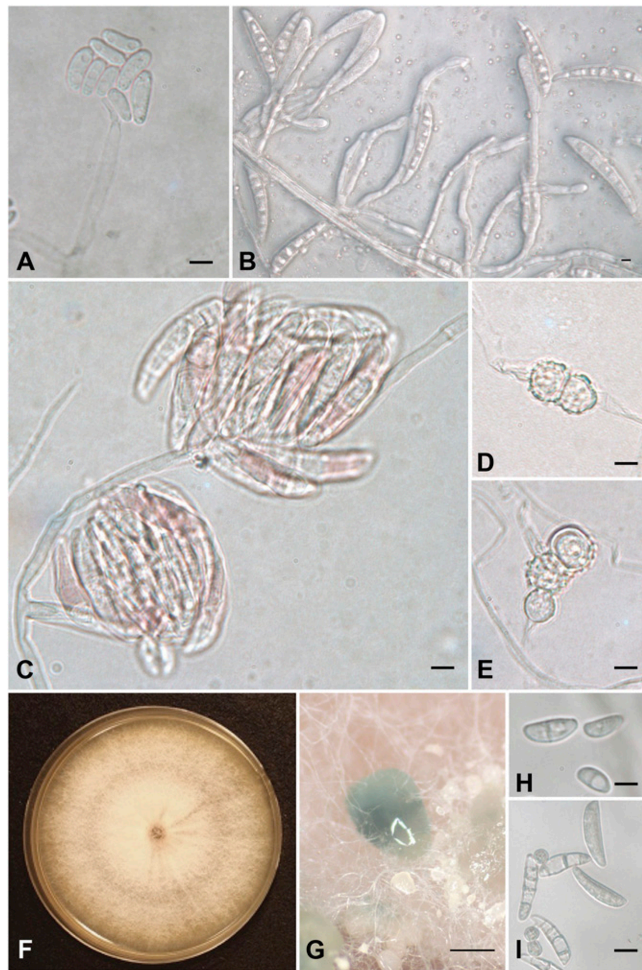

Fig. 3. A, B, D, I, Morphology of 14-day-old *F. kuroshium* cultures grown on synthetic low-nutrient agar (SNA) and **C, E, H, F, G,** on potato dextrose agar (PDA). **A,** Aseptate microconidium. **B,** Sporodochial conidiogenous philades forming fusiform-clavate macroconidia. **C,** Sporodochial conidiophores exhibiting reddish pigmentation. **D,** Paired rough-walled chlamydospores. **E,** Terminal rough-walled chlamydospores. **F,** Colony morphology on PDA at 2 weeks. **G,** Blue-green conidial masses formed in culture on PDA after 1 month. **H,** 0-1 septate microconidia. **I,** Multiseptate macroconidia with chlamydospore rough-walled formation. **A** to **E, H** and **I,** 5 µm; **G,** 1 mm.

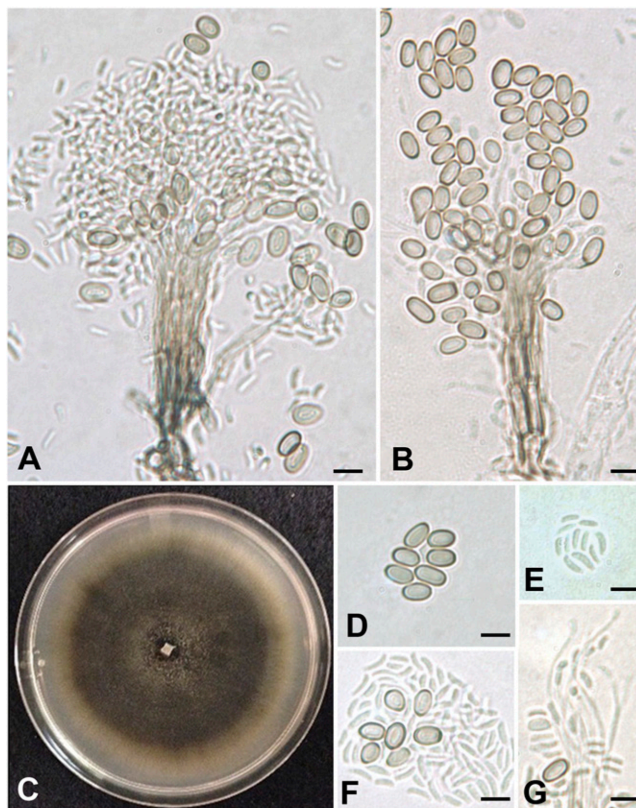

Fig. 4. A to **G,** Morphology of 14-day-old *Graphium kuroshium* cultures grown on potato dextrose agar (PDA). **A,** Synnema bearing cylindrical conidia with truncated ends. **B,** Synnema bearing ovoid conidia with thick walls. **C,** Colony morphology on PDA at 1 month. **D,** Ovoid conidia with thick walls. **E,** Cylindrical conidia with truncated ends. **F,** Both morphologically distinct conidia types. **G,** Conidiogenous philades forming on synnema. **A, B, D** to **G,** 5 µm.

light edges (1A). Reverse pigmentation is olive (3D3). Synnemata consist of packed parallel hyphae 1.36 ± 0.28 μm in width. Synnemata structures are 9.88 ± 2.24 μm wide at the top, 6.12 ± 1.98 μm wide at the base of the fruiting body. Abundant hyphae rhizoid in shape present at the base of synnemata. Two types of aseptate conidia are observed from synnemata structures; hyaline cylindrical conidia with truncated ends on PDA in the dark: 3.0 to 7.2 × 0.8 to 2.3 μm total range, 4.8 ± 0.8 × 1.4 ± 0.3 μm on average (ex type: 3.4 to 6.8 × 1.0 to 1.9 μm total range, 4.8 ± 0.8 × 1.4 ± 0.2 μm on average) and ovoid, thick walled, conidia on PDA in the dark: 3.4 to 5.6 × 1.8 to 3.6 μm total range 4.6 ± 0.4 × 2.8 ± 0.3 μm on average (ex type: 3.7 to 5.6 × 2.3 to 3.1 μm total range, 4.7 ± 0.5 × 2.8 ± 0.2 μm on average).

Known host range. Acer negundo, *Albizia julibrissin*, *Baccharis salicifolia*, *Baccharis pilularis*, *Dombeya cacuminum*, *Erythrina humeana*, *Persea americana*, *Populus fremontii*, *Populus nigra*, *Platanus racemosa*, *Quercus agrifolia*, *Quercus suber*, *Ricinus communis*, *Robinia pseudoacacia*, *Salix gooddingii*, *Salix laevigata*, *Salix lasiolepis*, *Tamarix ramosissima*.

Ex-type culture. CBS142643 = Francis Na UCR4593

Fungal distribution. San Diego County, California, U.S.A.

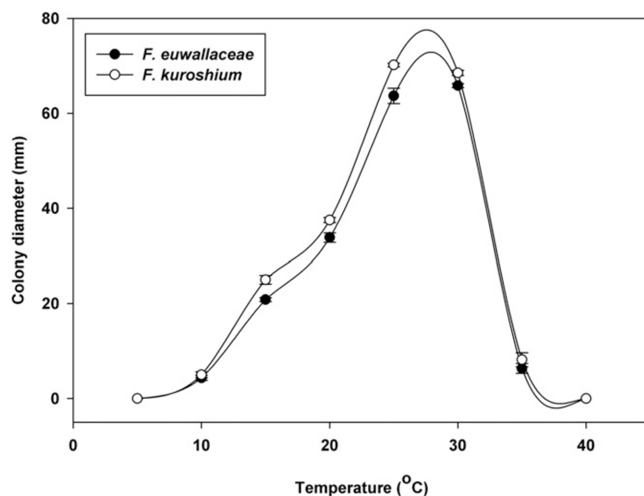

Fig. 5. Radial growth of *Fusarium kuroshium* and *F. euwallaceae.* Colony diameters of the resulting colonies were measured after 9 days of incubation. Vertical bars represent standard errors of the mean.

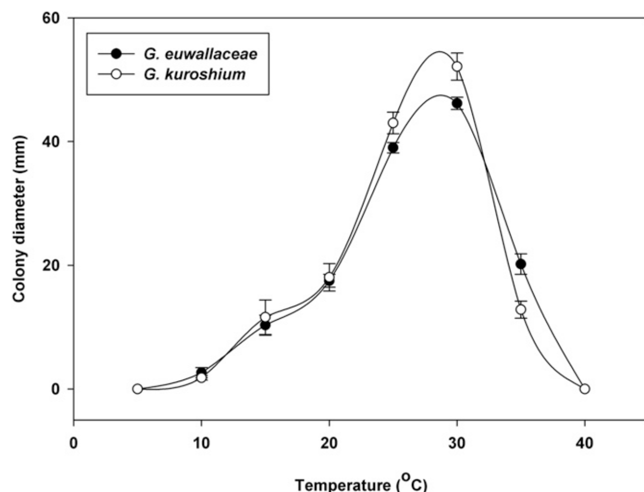

Fig. 6. Radial growth of *Graphium kuroshium* and *G. euwallaceae.* Colony diameters of the resulting colonies were measured after 14 days of incubation. Vertical bars represent standard errors of the mean.

Additional specimens examined. United States, California. Fallbrook, San Diego County. Surface of *Euwallacea* sp. galleries in *P. americana*, 2015, F. Na, UCR4594. United States, California. Bonsall, San Diego County. Surface of *Euwallacea* sp. galleries in *P. americana*, 2015, F. Na, UCR4606. United States, California. Bonsall, San Diego County. Surface of *Euwallacea* sp. galleries in *P. americana*, 2015, F. Na, UCR4607. United States, California. Bonsall, San Diego County. Head of *Euwallacea* sp. in infested *P. americana*, 2015, F. Na, UCR4608. United States, California. Bonsall, San Diego County. Head of *Euwallacea* sp. in infested *P. americana*, 2015, F. Na, UCR4609. United States, California. Escondido, San Diego County. Surface of *Euwallacea* sp. galleries in *P. americana*, 2015, F. Na, UCR4616. United States, California. Escondido, San Diego County. Surface of *Euwallacea* sp. galleries in *P. americana*, 2015, F. Na, UCR4617. United States, California. Escondido, San Diego County. Head of *Euwallacea* sp. in infested *P. americana*, 2015, F. Na, UCR4618. United States, California. Escondido, San Diego County. Head of *Euwallacea* sp. in infested *P. americana*, 2015, F. Na, UCR4622.

Comments. G. kuroshium colonies exhibit some morphological overlap to *G. euwallaceae*, an ambrosial *Graphium* species associated with polyphagous shot hole borer (*Euwallacea* sp. #1) (O'Donnell et al. 2015), in culture. Conidial dimensions of ovoid conidia produced by *G. kuroshium* slightly differ from those produced by *G. euwallaceae*, as the average length of ovoid conidia produced by *G. kuroshium* was lower than the minimum length observed in *G. euwallaceae* (Lynch et al. 2016). Hyaline cylindrical conidia with truncated ends dimensions were within the range of observed dimensions in *G. euwallaceae.* Phylogenetic analysis shows that *G. kuroshium* is closely related to, but genetically distinct from *G. euwallaceae.*

Fungal recovery from KSHB female heads. *F. kuroshium* was the most abundant fungal species from macerated heads of KSHB recovered from avocado. *F. kuroshium* colony forming units (CFUs) were found to be present at the highest relative abundance (83.5%) compared with *G. kuroshium* CFUs, which were present at a significantly lower abundance (16.5%) (Fig. 7). Seventy percent of the beetles examined contained both *F. kuroshium* and *G. kuroshium* colonies with the remaining 30% containing exclusively *F. kuroshium* colonies. From the sampled beetles, *G. kuroshium* was not found exclusively in any mycangia and no beetle was found to contain *Paracremonium pembeum*, which Lynch et al. (2016) reported to

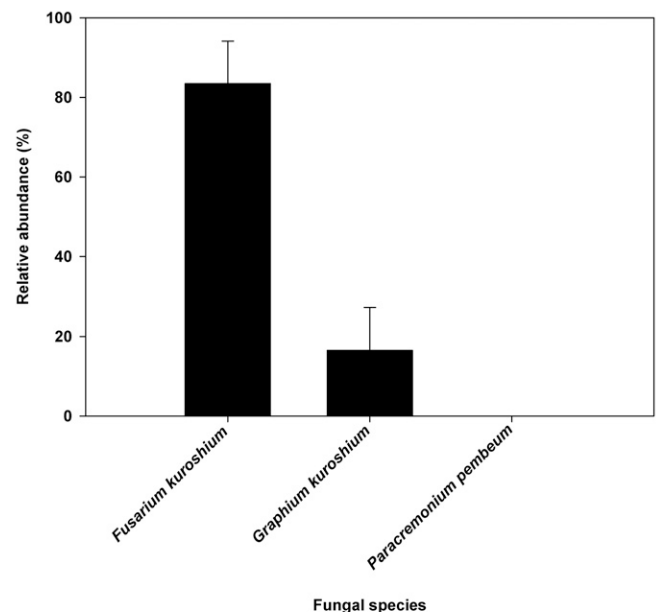

Fig. 7. Relative abundance of colonies *Fusarium kuroshium*, *Graphium kuroshium*, and *Paracremonium pembeum* recovered from KSHB female heads collected from avocado in San Diego County. Vertical lines represent standard error of the mean.

be a facultative inhabitant in the mycangia of PSHB. No inhibition between mutualists was observed on the spread plates.

Pathogenicity assay. Avocado (cv. Zutano) seedlings infected with *F. euwallaceae*, *F. kuroshium*, *G. euwallaceae*, or *G. kuroshium* did not show dieback symptoms during the length of the experiment; however, sugar exudate resulting from mechanical damage of the phloem caused by the inoculation procedure was observed in most avocado seedlings used in the experiment including control treatments. Mean lesion lengths (Fig. 8) caused by *F. kuroshium* were found to be significantly smaller from those caused by *F. euwallaceae* ($P < 0.001$). Moreover, both *Fusarium* spp. tested were found to produce lesions significantly greater compared with the control ($P < 0.01$). Avocado seedlings inoculated with *F. kuroshium* produced lesions with an average length of 3.15 ± 1.8 cm, and seedlings inoculated with *F. euwallaceae* produced lesions with an average length of 6.89 ± 2.1 cm. Mean lesion lengths in plants infected with *G. kuroshium* (Fig. 8) did not significantly differ from those in plants infected with *G. euwallaceae* ($P > 0.05$) but both were significantly greater than the control ($P < 0.001$). Plants infected with *G. euwallaceae* showed lesions with an average length of 6.4 ± 2.8 cm, and those infected with *G. kuroshium* showed lesions with an average length of 7.6 ± 4.1 cm. There were significant differences in lesion length ($P < 0.001$) from *F. kuroshium* compared with all other fungi inoculated, but it was still significantly greater than the control ($P < 0.05$). Control seedlings treated with sterile agar plugs produced a wound response with a mean length of 0.6 ± 0.1 cm. Fungal recovery for all species ranged from 90 to 100% and none of the inoculated fungi were recovered from the controls.

Discussion

Multigene molecular analyses of both *F. kuroshium* (Fig. 1) and *G. kuroshium* (Fig. 2) associated with KSHB reveal a distinct phylogeny compared with closely related AFC fusaria and *Graphium* species, respectively. PSHB and KSHB invading California are morphologically indistinguishable members of the *Euwallacea* genus, but have been previously found to be genetically distinct at the COI locus as well as other nuclear genes tested (O'Donnell et al. 2015; Stouthamer et al. 2017) and exclusively associated with distinct *Fusarium* spp. as well as *Graphium* spp. based on results from the current study. Although there is morphological overlap in AFC fusaria (Freeman et al. 2013; Kasson et al. 2013; O'Donnell et al. 2015) as well as in *Graphium* spp. (Lynch et al. 2016) associated with invasive SHB, molecular-based identification at informative loci such as TEF1-α, RPB-1, and RPB-2 is the best approach for proper identification of PSHB and KSHB symbionts. A PCR-based assay has already been described that can distinguish AFC fusaria (Short et al. 2017) and may be useful for diagnostic identification of these *Fusarium* spp. symbionts with sympatric geography. Based on morphological and molecular differences of these two fungal symbionts of KSHB, we propose the names *F. kuroshium* sp. nov. and *G. kuroshium* sp. nov. based on the exclusive association with the insect vector KSHB (*Euwallacea* sp. nr. *fornicatus*) in California. Both populations of fungal symbionts associated with PSHB and KSHB invasive to California are shown here to be pathogenic on avocado and were first observed in Los Angeles and San Diego counties, respectively, causing FD symptoms on 15 different host species (http://eskalenlab.ucr.edu/shotholeborerhosts.html). The two pest disease complexes KSHB-FD and PSHB-FD should be considered distinct, but collective threats spreading and adversely impacting woody hosts throughout California.

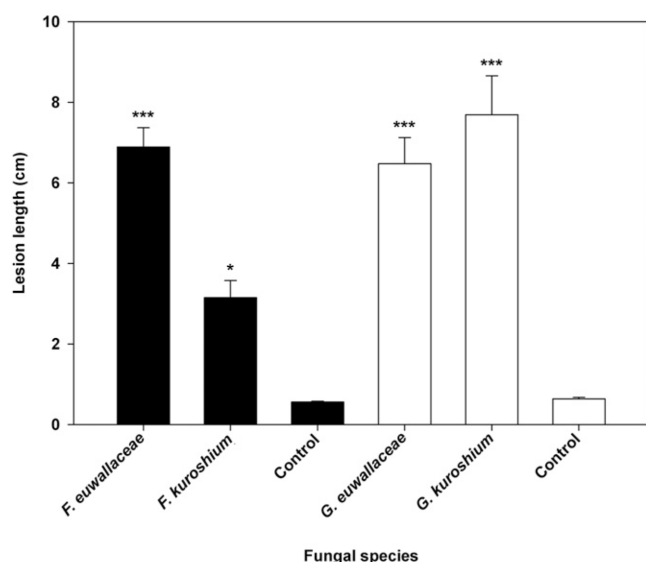

Fig. 8. Mean lesion length of xylem discoloration caused by *Fusarium euwallaceae*, *F. kuroshium*, *Graphium euwallaceae*, and *G. kuroshium* in 2-year-old avocado seedlings. Vertical lines represent standard error of the mean. *P*-values are represented as *** ($P < 0.001$), ** ($P < 0.001$), and * ($P < 0.05$) according to a Tukey's bootstrap test with confidence level $\alpha = 0.05$.

Ambrosia beetles from the *Euwallacea* genus have invaded the U.S. on multiple occasions (CABI 2015; Hulcr and Dunn 2011; Hulcr and Stelinski 2017; Kasson et al. 2013; O'Donnell et al. 2015; Stouthamer et al. 2017), carrying fungal symbionts that have evolved with them in mutualisms that provide the beetles with nutritional supplement from low-nutrient xylem tissue, while the fungi receive reliable dispersion and direct inoculation into plant hosts (Beaver 1989). The exclusive relationships are maintained upon invasion, which is seen in PSHB-FD as it has invaded California (Eskalen et al. 2013) and Israel (Mendel et al. 2012), vectoring the same previously described symbionts (*F. euwallaceae*; Freeman et al. 2013; *G. euwallaceae*, and *P. pembeum*; Lynch et al. 2016) in all locales. The present study suggests an exclusive relationship is also present in the KSHB-FD invasion of California with fungal mutualists *F. kuroshium* and *G. kuroshium* classifying KSHB-FD as a unique pest-disease complex. These invasive pest-disease complexes may also be similarly unique for other areas of the U.S. and other countries affected by invasive *Euwallacea* spp., but it is currently unknown to what extent these populations of invasive *Euwallacea* spp. can obtain new symbionts in what have been previously described as "host shifts" in which *Euwallacea* spp. AFC mutualists can change associations with the insect hosts over time (O'Donnell et al. 2015). In addition to AFC mutualists, other mutualists such as *Graphium* spp. may also undergo host shifts in areas where more than one population of *Euwallacea* spp. exists. PSHB and KSHB are currently both present in Orange County and parts of Los Angeles County (http://eskalenlab.ucr.edu/distribution.html) and present a situation in which potential "host shifts" may occur and should be investigated to find if new combinations of *Fusarium* spp. and *Graphium* spp. are possible and what effects they may have on severity of FD in plant hosts.

Fusarium and *Graphium* species from other *Euwallacea* spp. in southeast Asia included in this study allowed for investigation into the potential origin of the symbionts recovered from KSHB in California. *F. kuroshium* obtained from KSHB was shown to be closely related to some AFC isolates recovered from *Euwallacea* sp. in Taiwan (Fig. 1), yet distant from *F. euwallaceae* associated with PSHB from California and Israel. The placement of two AFC isolates (*Fusarium* sp. AF-13 Taiwan) originating from Taiwan with the closest relation to *F. kuroshium* suggests a potential origin of KSHB-FD based on phylogenetic relationship, corroborating previous findings from Stouthamer et al. (2017) where analysis of the cytochrome oxidase *c* subunit I (COI) mitochondrial gene in *Euwallacea* spp. confirmed KSHB haplotype (H22) found in California matched one of the four KSHB haplotypes found in Taiwan. *Fusarium* spp. AF-14 also show close relation to *F. kuroshium* and *F. euwallaceae* as they are resolved in AFC clade B (Kasson et al. 2013; O'Donnell et al. 2015) but form a distinct phylogenetic lineage along with *Fusarium* sp. AF-15 Taiwan, which is closely related to *Fusarium* sp. AF-4 from Pennsylvania, U.S.A. *Fusarium* sp. AF-16 Taiwan is notable as a new AFC species that forms a unique lineage distinct from previously described AFC species (Kasson et al. 2013; O'Donnell et al. 2015). The diversity present in Taiwan isolates included in this study

may be explained by greater diversity of host *Euwallacea* spp. in this geographic region and/or the coevolution as exclusive fungal mutualists of the vectors. Alternatively, AFC may associate with multiple *Euwallacea* spp. in Taiwan, creating opportunity for parasexual or potential sexual fungal interactions with other AFC fusaria present in the native habitat, increasing diversity. Further genomic sequencing and evolutionary analyses of populations of Taiwanese strains will allow for further testing of the relative contributions of these mechanisms to the observed genetic diversity.

The other common fungal symbiont of KSHB, *G. kuroshium*, was also shown to be genetically distinct from *G. euwallaceae* associated with PSHB using a two-gene phylogeny (Fig. 2, ITS, TEF-1α). Although we were unable to completely resolve the relationship of *G. kuroshium* to other *Graphium* spp. associated with *Euwallacea* spp. in Taiwan in the current study, as was possible for the AFC *Fusarium* spp., there is opportunity to elucidate phylogeographic patterns from this group in future studies by sampling from more native southeast Asian areas. Understanding the geographical origins of the ancestors of the fungi associated with invasive *Euwallacea* spp. may reveal relevant locations to narrow the search for natural control methods using natural predators, parasites, and other biotic/abiotic interactions that adversely affect these pests and can potentially provide an effective management strategy of these invasive pests.

In this study, the previously described fungal species, *P. pembeum* (Lynch et al. 2016), was not recovered from KSHB female heads and/or in wood tissue from galleries. This finding was notable because *P. pembeum* has previously been recovered with *F. euwallaceae* and *G. euwallaceae* from both the heads of the PSHB female beetles and their galleries (Lynch et al. 2016). The role of *P. pembeum* in the life cycle is unclear, but has been previously reported to be recovered at higher abundance from beetle larvae in castor bean and avocado than other hosts examined (Freeman et al. 2016). In this study, we only cultured fungi from adult females and gallery walls; we did not attempt to recover fungi from preadult stages of the life cycle, which may explain why we were not able to recover *Paracremonium* spp., but it should be investigated further to see if *Paracemonium* spp. are mutualists of KSHB.

F. kuroshium and *G. kuroshium* associated with the KSHB-FD complex were found to be pathogens on healthy young avocado plants and their status as a causal agent was confirmed by Koch's postulates performed in this study (Fig. 8). The symptom severity of *F. kuroshium* observed during the pathogenicity trial was significantly less ($P < 0.05$) when compared with *F. euwallaceae*, with the mean lesion size 46% of the size to that of *F. euwallaceae* over a 4-week time frame. Conversely, *G. kuroshium* was found to cause lesion sizes comparable to those of *G. euwallaceae*. The mode of ambrosia beetle damage in susceptible hosts from PSHB-FD and KSHB-FD is likely "Mode 2" suggested by Hulcr and Stelinski (2017), where mass accumulation of the pests on stressed trees leads to significant damage. Although both symbionts of KSHB are shown here as relatively weak pathogens, it is likely a combination of attacks by the beetle and inoculations of their mutualists that lead to FD symptoms, which can increase in severity when mass attacks accumulate on a susceptible host. In San Diego County, KSHB-FD has already been reported in commercial avocado groves and is causing branch dieback in multiple locations (S. Lynch, personal communication), but it is currently unknown what impact the pests will have on avocado production. Therefore, the pests should be monitored in these areas to gain a better understanding of the extent of damage caused in California. More plant species in urban forests and native vegetation than the agriculturally important commodity avocado are affected by this disease, as the reproductive host range of PSHB-FD has been previously described (49 hosts) (Eskalen et al. 2013; Mendel et al. 2012), but the host range of KSHB-FD is not yet fully resolved. More investigation into the full reproductive host range and the biotic and abiotic factors that contribute to host susceptibility and defense responses to PSHB-FD/KSHB-FD can aid in elucidating why these invasive pest-disease complexes are destructive in invaded areas as well as their native habitat.

The classification of KSHB-FD as a unique complex invading California distinguishes this new complex from the closely related PSHB-FD complex. There is more diversity among the fungal symbionts of ambrosia beetles previously reported in their native habitat (Beaver and Liu 2010; Hulcr and Cognato 2010; Li et al. 2015; Stouthamer et al. 2017). These invasive beetles present in California and other invaded areas are likely subsamples of a larger diverse Asian population. This may explain the apparent exclusive relationships that are found among fungal symbionts recovered from ambrosia beetles from the *E. fornicatus* complex in invaded areas. The extent of these exclusive symbiotic relationships in their native habitats is currently unknown and it may be possible that the beetles are associated with multiple species of AFC fusaria, *Graphium* spp., *Paracremonium* spp., and/or other unidentified mutualists. Since *Euwallacea* spp. are completely dependent on their mutualists to provide nutrition from nutrient-poor xylem tissue (Baker and Norris 1968; Beaver 1989), it would be informative to explore the pathogenicity and virulence of fungal symbionts associated with *Euwallacea* spp. in their native regions on susceptible hosts to investigate if there is any correlation to beetle fecundity. Although 95% of ambrosia beetle-fungal complexes are reported to be economically harmless (Hulcr and Stelinski 2017), biological information from these complexes, including species descriptions of both the insect and pathogen(s) involved in native and invasive SHB-fungi complexes, both destructive and nondestructive, would be highly informative. This information should extend past scientific literature and should be recognized by government agencies worldwide to regulate and restrict the movement of new, potentially destructive, SHB-fungi complexes into other uninfested areas outside of their native habitat.

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