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Identification of Two Novel Fungal Species Associated with Kuroshio Shot Hole Borer (*Euwallacea* sp.) and Evaluation of Novel Biological Control Method to Inhibit the Fungal Associates of the Invasive Ambrosia Beetle Species in California

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by

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General introduction

Ambrosia beetles are wood-boring insects belonging to the subfamilies Scolytinae and Platypodinae within the Curculionidae family. Ambrosia beetles are known to possess two defining characteristics of ambrosia beetles: construction of galleries in xylem tissues of woody plant species, and fungiculture within these galleries. These characteristics have aided the invasion of an ambrosia beetle species named polyphagous shot hole borer (*Euwallacea* sp.) in California, which has prompted the research in this thesis.

Ambrosia beetles possess powerful mandibles that allow them to chew through woody tissues, enabling them to construct networks of tunnels in the xylem tissues of host plant species. These tunnels serve as the beetles' nests, offering effective protection from the environment, parasitoids, and predators (Kirkendall, Biedermann, & Jordal 2015). Many ambrosia beetle species can carry out most of their life cycles within these well-protected galleries, remaining protected until the newly emerged adult beetles exit the maternal galleries to create their own galleries. Many are not exposed to the environment for very long after emergence either – as xyleborine ambrosia beetles, the adult females of the ambrosia beetle species polyphagous shot hole borer (*Euwallacea* sp.) reproduce by sib-mating in the galleries, thus the females are already mated and fully capable of creating a beetle gallery of its own as soon as they emerge from maternal galleries (Smith and Hulcr, 2015). Life in these galleries is aided by the cultivation of ambrosial fungus species inside the galleries. During the construction of the galleries, the ambrosia beetles deposit conidia of mutualistically associated ascomycete ambrosia fungi

along the gallery surface (Batra, 1963). This process is facilitated by a specialized organ named mycangia, which stores the fungal conidia within its many cuticular invaginations and pits for future use during gallery construction (Beaver, 1989). These fungi colonize the host xylem tissues, producing mycelium and conidia that serve as the beetles' sole source of nutrients. This allows the beetles to obtain the nutrients needed for survival as the ambrosia fungi help concentrate the essential nutrients from the plant tissues. The beetles' "fungal gardens" are known to be actively maintained by these beetles via antimicrobial secretions or fungal cropping, as overgrowth of the fungi in the galleries can be fatal to the immobile eggs and pupae (Biedermann and Taborsky 2011).

These characteristics have allowed the ambrosia beetles to be successful as wood-boring insects, as the ability to colonize xylem enables the beetles to utilize a niche that is not commonly exploited by many other insects (Lindgren and Raffa 2013), while the fungiculture in galleries allows the beetles to remain protected throughout most of their life cycles. This adaptability also allows the beetles to become invasive in nonnative habitats. The invasion of polyphagous shot hole borer in California demonstrates the ambrosia beetle's adaptability: since the discovery of the invasive ambrosia beetle species in Los Angeles County in 2012, the beetles have since spread to various urban landscapes and natural habitats in multiple counties of Southern California, threatening various native and nonnative woody plant species that occur in these locations (Eskalen *et al.,* 2013). While many ambrosia beetles and their associated ambrosia fungal species are generally considered to be decomposers of dying of dead wood (Batra, 1967), exceptions such as the redbay ambrosia beetles (*Xyleborus glabratus* Eichoff) (Fraedrich

et al., 2008; Hanula *et al.*, 2008), tea shot hole borer (*Euwallacea fornicatus*) (Danthanarayana, 1968), and polyphagous shot hole borer (*Euwallacea* sp.) (Eskalen *et al.* 2013) exist, which are capable of colonizing and killing living hosts.

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Chapter 1:

Identification of Two Novel Insect-Vectored Fungal Species *Fusarium kuroshium* sp. nov. and *Graphium kuroshium* sp. nov. Associated with Kuroshio Shot Hole Borer (*Euwallacea* sp.)

Abstract:

The invasive ambrosia beetle species polyphagous shot hole borer (*Euwallacea* sp., PSHB) and its ambrosial fungal associates have been studied extensively since the beetle's discovery in the Los Angeles County in 2012. Another ambrosia beetle species closely related to the polyphagous shot hole borer has been discovered in San Diego County (Kuroshio shot hole borer, KSHB), and the ambrosial fungal associates of this newly discovered beetle species have been found to be distinct from the fungal associates of the PSHB. The KSHB-associated *Fusarium kuroshium* sp. nov. and *Graphium kuroshium* sp. nov. have been found to be genetically distinct from *Fusarium euwallaceae* and *Graphium euwallaceae* in multilocus phylogenetic analyses of combined internal transcribed spacer region (ITS), elongation factor-1 alpha (EF-1α), and RNA polymerase 1 & 2 (RPB1, RPB2) sequences. Morphological comparisons between the fungal associates of KSHB and PSHB showed that *F. kuroshium* colonies were morphologically distinct from those of *F. euwallaceae,* while the sizes of all fungal structures examined were within observed dimensions of those found in *F. euwallaceae. G. kuroshium* colonies were found to be morphologically identical to *G. euwallaceae*'s, while the conidial dimensions of *G. kuroshium* microconidia were slightly different from

G. euwallaceae microconidia. The severity of symptoms caused in avocado (*Persea americana*) by *F. kuroshium*, *F. euwallaceae*, *G. kuroshium,* and *G. euwallaceae* were compared during a greenhouse pathogenicity trial. Symptom severity of *F. kuroshium* was found to be significantly lower compared to those caused by *F. euwallaceae* infection, while no difference in symptom severity was detected between the *Graphium* associates of KSHB and PSHB.

INTRODUCTION

Polyphagous Shot Hole borer (*Euwallacea* sp., "PSHB") is an invasive ambrosia beetle that is a threat to many hardwood species in Southern California (Eskalen *et al.* 2013). This invasive beetle species is known to cause potentially deadly dieback symptoms in its hosts as it infects the host plants with pathogenic fungal species during colonization. PSHB is known to be closely related to the tea shot hole borer (*Euwallacea fornicatus* Eichoff) found in India and Sri Lanka (Danthanarayana 1968), where it is known to be a serious pest of tea (*Camellia sinensis*). These ambrosia beetle species' similarities most notably show in their host preferences; *E. fornicatus* is known to be one of the few species of ambrosia beetles that attack and colonize healthy plants as opposed to other ambrosia beetle species' usually stressed or dead plant hosts (Batra 1967, Hulcr et al. 2011), and polyphagous shot hole borer shares this trait. These beetles' preference to colonizing living host plants is considered to be highly unusual. The change in an invasive ambrosia beetle's host preference when introduced to a new niche has been documented to be attributed to "olfactory mismatch", in which the invading beetles are

improperly recognize local plant-emitted compounds to be those emitted by stressed or dead trees in its native ranges (Hulcr *et al.,* 2011). In addition to this versatility that allows for greater availability of suitable host plants, PSHB also possesses an extremely wide host range. In California, PSHB has been observed to attack a wide range of tree species –139 species of hardwood species are known to be susceptible to beetle attacks, of which 41 are reproductive hosts that allow the beetles to complete their life cycles (Eskalen *et al.*, 2013, *http://eskalenlab.ucr.edu/avocado.html*). The wide host range of PSHB includes common landscape trees such as the California sycamore (*Platanus racemosa*) and coast live oak (*Quercus agrifolia*), as well as the commercially important crop avocado (*Persea americana*) (Eskalen *et al.,* 2013). This wide host range and the ubiquitous distribution of host plants in the Southern California have allowed Polyphagous Shot Hole Borer to proliferate rapidly into the neighboring counties surrounding the Los Angeles County since the beetle's discovery in 2012.

PSHB was discovered in 2012 when the invasive beetles and their fungal associates were found to have colonized over 100 species of hardwood plants in Southern California; this same beetle species was discovered in 2012 causing significant damage to the Israeli avocado groves (Mendel *et al.*, 2012, Eskalen *et al.,* 2013). Some of the most severely impacted areas in Israel due to beetle infestations were in commercial avocado groves, in which extensive physical damages to the trees were observed (Mendel *et al.*, 2012). The polyphagous shot hole borer's wide host range, coupled with the physical damage to tree hosts resulting from beetle colonization, became a critical concern to the California avocado industry in 2012. Beetle infestations were observed to be radiating out

from urban areas of Los Angeles County towards commercial avocado groves located in nearby counties of Ventura, Orange, and San Diego Counties (Lynch *et al.,* 2016). As of 2016, the beetles can now be found in many urban landscape trees in Los Angeles, Orange, Riverside, San Bernardino, and San Diego Counties, in several avocado groves located in Los Angeles, Orange, Ventura, and San Diego Counties, and even among the native tree species in the Angeles National Forest. Without current, effective methods to eliminate the beetles or treat the symptoms caused by the fungal associates, the need for a better understanding of the polyphagous shot hole borer and its fungal symbionts, and development of effective management strategies against this pathogen-vector complex is of paramount importance.

Like other ambrosia beetle species, PSHB colonizes the plant hosts by boring networks of galleries in the xylem tissues of the hosts (Eskalen *et al.,* 2012). These galleries serve as lairs for the beetles, in which the beetles cultivate species of symbiotic fungi that help concentrate the nutrients from the plant tissues. This cultivation of symbiotic fungi is facilitated by specialized organs in the heads of the beetles named mycangia, which are used to store fungal conidia of the symbiotic fungi. The conidia are stored in the mycangia as the beetles and their young feed on the mycelia that grow from the gallery surface, allowing the future generations of the beetles to vector the symbiotic fungi to other locations as they emerge from the galleries as adults. This dispersal method is currently the only known for the beetle-symbiotic fungal species. Three species fungi are associated with the Polyphagous Shot Hole Borer – *Fusarium euwallaceae*, *Graphium euwallaceae*, and *Paracremonium pembeum* (Freeman 2013, Lynch 2016).

While providing mycelial masses for the beetles to consume on the gallery surface, these fungi also infect and colonize the host xylem vessels, restricting water and nutrient movement in plants. The combination of the mechanical damage caused to the xylem by the beetles' boring during the gallery formation and the occlusion of xylem vessels caused by the fungal infection can lead to a potentially fatal dieback symptom in host plants, which has been named the Fusarium Dieback. Extremely severe infestations in landscape trees can even create safety hazards when the structural weakening from the dieback cause heavily infested tree branches to break off and fall from trees.

Though the distribution of all currently known beetle-infested areas in Southern California shows infestations in Los Angeles, Orange, Riverside, Ventura, and San Diego Counties, San Diego County was not known to be infested prior to 2013. In 2013, new beetle infestations were detected in landscape trees located in San Diego County. This beetle infestation was initially considered to be a new extension of the existing infestation in Orange County, as the beetles and the fungal symbionts obtained from the newly discovered location were morphologically identical to the PSHB and its fungal symbionts. However, preliminary DNA analysis of the fungal species obtained from the new location revealed significant differences between these isolates and those obtained from any other known infested areas in Southern California. This finding suggested that the San Diego infestation may be the result of a separate invasion event involving a related but distinct pathogen-vector complex. This hypothesis was further supported by the similar results obtained from DNA analysis of the beetles collected from the San Diego infestation by Stouthamer *et al.* which showed that the beetles from San Diego

County were also genetically distinct from those found in Los Angeles County (Cooperband *et al.* 2016; Richard Stouthamer, Univ. of California Riverside, personal communication). This newly discovered beetle species was named the kuroshio shot hole borer (KSHB) in 2015. In order to elucidate the differences between the fungal symbionts of the kuroshio shot hole borer (*Euwallacea* sp. #5) (O'Donnell *et al.* 2014) and those of the polyphagous shot hole borer (*Euwallacea* sp.), further studies in characterizing these new fungal symbionts are needed.

In this study, we present morphology, genetic analysis, and pathogenicity data of KSHB-associated *Fusarium kuroshium* sp. nov. and *Graphium kuroshium* sp. nov., and compare these data with those of *Fusarium euwallaceae* and *Graphium euwallaceae* to describe the KSHB-associated fungal species as a novel species. The differences found in morphology, genetics, and symptom severity between the PSHB-associated fungi and KSHB-associated fungi support the hypothesis that two invasion events likely originating from geographically separate locations involving two related but genetically distinct vector-pathogen complexes have occurred in Southern California. We propose the names *Fusarium kuroshium* for the KSHB-associated *Fusarium* species and *Graphium kuroshium* for the KSHB-associated *Graphium* species.

MATERIALS AND METHODS

Fungal isolate collection. - Fungal isolates used in this study were obtained from the heads of beetles or from the surfaces of their galleries in their reproductive host tree, using methods described by Eskalen *et al.* (2013). 40 beetles were obtained from infested

Persea americana and *Platanus racemosa* in 4 separate sampling areas in San Diego County: Bonsall, CA, El Cajon, CA, Escondido, CA, and Fallbrook, CA. 10 beetles and 10 gallery samples were collected from each location. Beetles obtained were surface sterilized by submerging in 70% ethanol, and their heads were separated from the thoracic and abdominal segments, which were then macerated in 1.5 ml Eppendorf tubes with sterile polycarbonate pestles. The macerated heads were suspended in 500 µl of sterile water and serially diluted the suspensions to 50%, 10%, 5%, and 1% of the original concentration. 50 µl of the suspensions were pipetted onto petri plates containing potato dextrose agar (PDA) amended with tetracycline and spread using sterile glass rods. Fungal colonies were isolated from these plates after 5 days of incubation in 25°C, and 30 plates spread with undiluted beetle head suspensions were randomly selected to observe for relative abundances of each fungus similarly to methods described by Lynch *et al.* (2013). Beetle gallery samples were obtained by carving out small pieces of galleries from infested sycamore (*Platanus racemosa*) and avocado (*Persea americana*) wood and sampling the gallery surfaces by scraping with a sterile pick (Kajimura *et al.*, 1992). Picks containing the wood scrapings were washed with 1ml of sterile water, and 50 µl of the wash were pipetted onto plates containing PDA amended with tetracycline and spread using sterile glass rods. Plates were incubated in 25°C for 5 days, and Individual fungal colonies were isolated from the plates.

Fungal DNA Extraction, PCR, and Phylogenetic Analysis. - To determine the genetic relatedness between the *Fusarium* and *Graphium* isolates obtained from various locations in which PSHB and KSHB-related species of *Euwallacea* sp. occur, multilocus

phylogenetic analysis was conducted. DNA sequences from 10 isolates of *Fusarium kuroshium,* 10 isolates of *Graphium kuroshium*, 10 isolates of *Fusarium* sp. from Taiwan (provided by Dr. Chi-yu Chen), 3 isolates of *Fusarium ambrosium* from India and Sri Lanka, and 3 additional isolates of *Fusarium* spp. from GenBank as outgroup were used in the phylogenetic analyses. We extracted the genomic DNA of the fungal isolates obtained from beetles and gallery samples by using a modified DNA extraction protocol by Cenis *et al.* (1992). 50 ug of fungal mycelia harvested from 7-day old *F. kuroshium* or 14-day old *G. kuroshium* pure cultures were added to microtubes 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, USA) to lyse. 150 µl of sodium acetate was then added to each microtube, and the microtubes were placed in a -20°C freezer for 10 minutes. The microtubes were centrifuged at 14,000 RCF, then the supernatants were mixed with isopropanol prior to storing in 0°C for 10 minutes. The precipitated DNA was pelleted by centrifugation at 14,000 RCF, air dried, then suspended in TE buffer.

DNA sequences of the internal transcribed spacer (ITS), elongation factor-1 alpha (EF1α), RNA polymerase 1 & 2 (RPB1, RPB2, *F. kuroshium* only) were selected for multilocus phylogenetic analysis of the *F. kuroshium* and *G. kuroshium* Oligonucleotide primers ITS4 and ITS5, EF1 and EF2, EF1F and EF2R (for EF1α, *Fusarium* and *Graphium*, respectively), F5 and R8, F7 and G2R (for RPB1), 5F2 and 7CR, 7CF and 11AR (for RPB2) were used in PCR amplification of ITS1-5.8S-ITS2 (White *et al.*, 1990), Gräfenhan EF1-α (Jacobs *et al.*, 2004), RPB1-1 and RPB1-2 (O'Donnell *et al.*,

2010), RPB2-1 and RPB2-2 (O'Donnel *et al.*, 2007) respectively. 2 pairs of primers were used for the amplification of two overlapping regions within RPB1 (O'Donnell *et al.,* 2010), and the sequences were combined in the software Sequencher 4.6 (Gene Codes Corp.). RPB2 sequences were obtained using the same method. Each PCR reaction mixture consisted of 12.5 µl GoTaq DNA Polymerase (Promega), 9.3 µl sterile DNase-free water, 0.6 µl of 10x forward primer, 0.6 µl of 10x reverse primer PCR, and 2 µl of genomic DNA template to a total of 25 µl reaction mixture. PCR was performed using published cycling parameters (White *et al.,* 1990; O'Donnell *et al.,* 2007; O'Donnell *et al.,* 1998; Jacobs *et al.,* 2004). Amplified products were separated by gel electrophoresis in 1% agarose gel with 0.5x Tris-boric acid-EDTA buffer, stained with SYBR Green (Invitrogen), then viewed under UV. Products were purified using ExoSAP-IT (Affymetrix) then sequenced in both directions at the Institute for Integrative Genome Biology, University of California Riverside with corresponding primers.

DNA sequences were edited and combined using Sequencher 4.6 (Gene Codes Corp.), and the sequences were aligned using ClustalX (Thomson *et al.,* 1997). The multilocus phylogenetic analysis was conducted using MEGA 6 software (Tamura *et al.,* 2013). The analysis of the *Fusarium* samples was performed using sequence sets created with the sequences of ITS, EF-1a, RPB1, and RPB2, and the analysis of the *Graphium* samples was performed using data sets created with the sequences of ITS, and EF-1a. The sequences from the selected loci were combined together manually in software prior to performing the phylogenetic analysis. Maximum-parsimony, maximum-likelihood, and neighbor-joining methods were used to generate multiple phylogenetic trees (Trees made

using maximum-parsimony and neighbor-joining methods not shown). Gaps in sequences were considered to be complete deletions, and bootstrap values were calculated from 1,000 bootstrap replications. Other *Fusarium* isolates used in the analysis, including *F. euwallaceae*, *F. ambrosium*, and other species belonging in the *Fusarium solani* complex, were selected from the isolates used during the analysis of *F. euwallaceae* by Freeman *et al*. (2013). The sequences of *G. kuroshium* isolates were compared to those of *G. euwallaceae*, ambrosia beetle-associated *Graphium* spp. from USA (Pennsylvania), Vietnam, and Thailand, and other GenBank-available species of *Graphium* including bark beetle-associated fungi. All sequences used to compare with those of *G. kuroshium* isolates were selected from a published phylogenetic analysis of *G. euwallaceae* (Lynch *et al*. 2016).

Morphological characterization. - Morphological characteristics of the *Fusarium kuroshium* and *Graphium kuroshium* were observed using Olympus BX40 compound microscope with a Leica DFC420 camera attachment. Ten isolates of each fungal species were selected for use in the morphological observation. *F. kuroshium* cultures were grown on potato dextrose agar and synthetic low-nutrient agar (SNA) (Nirenberg *et al.*, 1976) under constant light for 14 days in 25°C. *G. kuroshium*. cultures were grown on potato dextrose agar and oat meal agar (OMA) (Gams *et al.,* 1998) under constant light for 14 days. The lengths and widths of the *F. kuroshium* and *G. kuroshium*. conidia were measured using SPOT Imaging Software, which was calibrated using the same camera-microscope combination prior to measuring.

Growth rates of *F. kuroshium* and *G. kuroshium* were obtained by measuring the fungal colony diameters in cultures incubated in various temperatures ranging from 5°C to 40°C. Plugs of agar containing the fungi were obtained from the edges of 1-week old cultures and placed on petri plates containing potato dextrose agar. 3 replicates of each fungal isolate were grown in incubators with set temperatures of 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, and 40°C. Diameters of the *F. kuroshium* colonies were measured every day for 9 days, and *G. kuroshium*. were measured every 2 days for 14 days. Experiment was repeated to a total of 2 trials.

Pathogenicity Assay. - Pathogenicity assays were conducted in greenhouse, in which 100 avocado seedlings were inoculated with *Fusarium euwallaceae, Fusarium kuroshium*, *Graphium euwallaceae,* or *Graphium kuroshium*. using similar methods described by Twizeyimana *et al.* (2013). Two isolates each from *F. euwallaceae, F. kuroshium*, *G. euwallaceae,* and *G. kuroshium* were chosen for use in the pathogenicity assay, and each fungus was used to infect 10 plants across 2 trials. The fungal cultures used during this experiment were grown on PDA for 7 days (*F. euwallaceae, F. kuroshium*) or 14 days (*G. euwallaceae, G. kuroshium*) in 25°C, and agar plugs 5mm in diameter were cut from the cultures with a sterile cork borer. We inoculated 100 2-year old avocado seedlings (cultivar: Zutano) with the agar plugs containing the *Fusarium* or *Graphium* isolates to observe their pathogenicity in avocado. The 5 mm-diameter cork borer used to cut the agar plugs was flame-sterilized then used to create openings of identical sizes compared to the agar plugs in the avocado stems, removing the phloem tissues and exposing the wood tissues for the pathogens to colonize. These wounds were filled with the agar plugs

containing the pathogenic fungi mycelium-side down then wrapped with Para film. Control plants were similarly wounded with a cork borer and wounds were filled with sterile agar plugs. Five avocado seedlings were used per fungal isolate and control in each trial, 2 isolates per fungal species, and the experiment was repeated for a total of 2 trials. The inoculated plants were incubated for 30 days in greenhouse prior to processing in lab.

The wood tissues of the inoculated plants were examined for visible lesions originating from points of fungal inoculation, and xylem samples from the margins of the lesions were taken to recover the pathogens from the plants. Lesion lengths were measured from the edges of visible discoloration in wood and recorded. Statistical analysis of differences in average lesion lengths produced by *Fusarium euwallaceae* and *Fusarium kuroshium* was conducted via nonparametric Wilcoxon matched pairs test at $\alpha = 0.05$. Xylem samples were collected from the margins of lesions, 1cm away from the margins, and 2cm away from the margins to confirm the presence of the inoculated fungi. All xylem samples collected from each plant were embedded in a petri plate containing potato dextrose agar amended with tetracycline, and the plates were incubated at 25°C for 5 days. Presence of *Fusarium* or *Graphium* from each xylem sample on plates was confirmed visually then recorded.

Statistical analyses – Analyses of xylem lesion sizes were performed using JMP 11.0.0 (SAS Institute Inc., Cary, North Carolina). Regression modeling was used to compare the mean colony sizes of *F. kuroshium* and *F. euwallaceae*, and same procedure was used to compare the mean colony sizes of *G. kuroshium* and *G. euwallaceae*. No significant

effect in colony size by fungal species was observed for both *Fusarium* and *Graphium* species.

Regression modeling was used to compare the sizes of lesions resulting from *F. euwallaceae* and *F. kuroshium* inoculations in *P. americana* stems. Significant effect was observed between the lesions caused by *F. euwallaceae* and *F. kuroshium* ($p<0.0001$). Lesion length data were transformed using log transformation to achieve normality, and normality was checked using Shapiro-Wilk test. The transformed data was compared by Tukey's honest significant difference (HSD) test at $\alpha = 0.05$. This process was repeated for the comparison of lesions resulting from *G. euwallaceae* and *G. kuroshium.*

RESULTS

Fungal isolate collection. - *Fusarium kuroshium* was the most commonly occurring fungal species in PDA plates spread with water suspensions containing macerated beetle heads. A total of 2,385 colonies of *Fusarium kuroshium* was counted across 30 plates spread with undiluted suspensions, while 428 colonies of *Graphium kuroshium* was counted among the same 30 plates. No inhibition between colonies was observed. 21 plates contained both *F. kuroshium* and *G. kuroshium* colonies, and 9 plates contained only *F. kuroshium* colonies. No plate was found to contain only *G. kuroshium* colonies, and no plate was found to contain *Paracremonium pembeum*. This absence of *A. pembeum* across the 30 plates was also reflected across all PDA plates spread with the beetle head suspensions in general as well.

Fungal DNA analysis. - Multilocus phylogenetic analysis using ITS, EF1-α, RPB1, and RPB2 sequences of *Fusarium* isolates revealed that *Fusarium kuroshium* belongs in a separate lineage from *Fusarium euwallaceae* with high bootstrap support (96%) (Fig. 1.1). All statistical methods (maximum likelihood, maximum parsimony, neighbor-joining) used during the analyses resulted in phylogenetic models with similar topologies and bootstrap values. The first clade containing *F. kuroshium*, *F. euwallaceae*, and shot hole borer-associated *Fusarium* species from Taiwan (Sequences provided by Dr. Chi-yu Chen) contained 5 major lineages: first lineage with all isolates of *F. kuroshium* (UCR3653~3662) and two isolates of *Fusarium* isolates from Taiwan (UCR4674, UCR4675), second lineage with *Fusarium euwallaceae* isolates only (NRRL 54722~54726), third lineage with 5 *Fusarium* isolates from Taiwan (UCR 4673, 4676, 4677, 4678, 4680), and the fourth lineage with 2 *Fusarium* isolates from Taiwan (UCR4672, 4681), and fifth with a single isolate from Taiwan (UCR4679). The second clade contained *Fusarium ambrosium* from India and Sri Lanka (100% bootstrap support), and the third clade contained 3 isolates of *Fusarium* spp. as outgroup (100% bootstrap support).

Within the clade containing all isolates of *Fusarium kuroshium and Fusarium* spp. from Taiwan, the first clade (96% bootstrap support) represents all *Fusarium kuroshium* isolates used in the analysis and two *Fusarium* sp. I isolates from Taiwan. Within this clade were 2 lineages, one of which contained all *Fusarium kuroshium* isolates (97% bootstrap support) while the other contained the *Fusarium* sp. I isolates from Taiwan (99% bootstrap support). The presence of the two Taiwanese *Fusarium* sp.

isolates within the clade containing *F. kuroshium* suggests that the *Fusarium* associate of KSHB is the most closely related to the *Fusarium* sp. associate of *Euwallacea* sp. found in Taiwan compared to all other *Fusarium* spp. observed. The second clade (85% bootstrap support) represents all isolates of *Fusarium* sp. obtained from *Euwallacea* sp. in Taiwan except the two *Fusarium* sp. I isolates found in the first clade. Within this clade are 2 subclades, first of which contain 2 *Fusarium* sp. II isolates (66% bootstrap support), second of which contains another pair of *Fusarium* sp. II isolates (99% bootstrap support). One *Fusarium* sp. II isolate did not clade with either of the two subclades. Third clade (85% bootstrap support) contained all *Fusarium euwallaceae* isolates used in this analysis. Sequences of these isolates were obtained from GenBank, including that of the isolate designated as the *Fusarium euwallaceae* type specimen (NRRL54722) (Freeman *et al.,* 2013). Fourth clade contained two isolates of *Fusarium* sp. III from Taiwan (97% bootstrap support), and the fifth clade contained a single isolate of *Fusarium* sp. IV from Taiwan (61% bootstrap support). Clade containing *Fusarium ambrosium* isolates from Indian tea shot hole borer beetles (*Euwallacea fornicatus*) was separated from all isolates mentioned above.

Similarly to *Fusarium kuroshium*, *Graphium kuroshium* was also found to be genetically distinct from *Graphium euwallaceae*. The multilocus phylogenetic analysis involving the internal transcribed spacer (ITS) and elongation factor (EF1-α) resolved the ambrosia beetle-associated *Graphium* isolates into 7 different lineages, one of which was corresponding to all *Graphium kuroshium* isolates. As observed previously in the study by Lynch *et al.* (2016), the 6 lineages excluding *G. kuroshium* corresponded to *Graphium*

euwallaceae isolates, *Graphium carbonarium* isolates from Vietnam, *Graphium carbonarium* isolates from China, *Graphium* sp. isolates from Pennsylvania (USA), *Graphium* sp. isolates from Thailand, and *Graphium* sp. isolates from Vietnam. The phylogenetic analysis placed all *G. kuroshium* isolates in clade with moderate bootstrap support (72%) that was separated from a similarly moderately supported clade containing all *G. euwallaceae* isolates (73%). Overall tree topology was comparable to that obtained by Lynch *et al.* (2016), except the lineage containing the Pennsylvania *Graphium* sp. isolates was placed in a well-supported clade outside of that containing all other ambrosia beetle-associated *Graphium* species. Sequence alignment between the ITS and EF1-α sequences from all *Graphium* isolates involved in this study showed that the *G. kuroshium* sequences differed from those of other *Graphium* isolates by several single base pair substitutions in both loci and a 9-bp in-frame deletion within the EF1-α gene.

Morphological characterization. - *Fusarium kuroshium* grown on PDA in 25°C are initially white in color, and colony color changes to greyish red with white margins in 14 days. Color darkens to greyish ruby with age. Cultures grown on SNA formed colonies with no pigmentation and produced abundant aerial mycelium. Reverse pigmentation of colonies grown on PDA were brownish red. Colony color differed from *Fusarium euwallaceae*, which produce darker reddish color, even in younger colonies. Conidia are produced on long aerial conidiophores, fusiform and clavate in shape. Septation within conidia occurs over time as the conidia mature; up to 5 septa per conidia were observed in 14-day old cultures. Conidia produced in 14-day old *F. kuroshium* cultures varied both in size and in the number of septa present in conidia. Cultures grown in relatively

nutrient-rich potato dextrose agar contained larger proportions of conidia with higher numbers of septa compared to those grown in SNA agar, though conidia with the same number of septa did not differ in size regardless of the media used for cultures (Fig. 1.3.1). Septation in conidia ranged from 0 to 5 septa in PDA cultures, and 0 to 4 septa in SNA cultures. Mean dimensions of *F. kuroshium* conidia in PDA by the number of septation were: no septation – 9.63 ± 2.98 x 3.45 ± 0.81 μm; 1-septate – 13.31 ± 3.23 x 4.42 ± 0.90 μm; 2-septate – 24.68 ± 5.70 x 6.78 ± 1.12 μm; 3-septate – 28.00 ± 3.99 x 7.18 ± 1.15; 4-septate – 34.87 ± 4.14 x 8.18 ± 1.12; 5-septate – 32.98 ± 0.66 x 7.67 ± 1.09. Conidia in SNA cultures: no septation – 9.47um x 3.39um; 1-septation – 13.16 μm x 4.34 μm; 2-septation – 26.05 μm x 7.34 μm; 3-septation – 28.24 μm x 7.52 μm; 4-septation – 36.39um x 9.11 μm. Conidia with no septation was the most common in both PDA and SNA cultures, followed by 1-septate conidia in SNA and 3-septate conidia in PDA.

G. kuroshium grown on PDA in 25°C are initially white, and colony changes in color to dark green with yellowish white margins in 14 day old cultures. The colony color darkens over time. Similar coloration was observed in colonies grown on oat meal agar. Aerial mycelium was septate and hyaline, and only observed on cultures grown on PDA that were incubated in 25°C or 30°C. Colonies on PDA cultures incubated in 10°C, 15°C, and 35°C were irregular in shape, pale yellow-colored, and mucoid in texture. Colonies on OMA were circular and flat. Abundant microconidia and macroconidia production was observed from their respective synnemata. Macroconidia are aseptate, thick-walled and symmetrical ellipsoid in shape, microconidia are aseptate, cylindrical in shape with

truncated ends (Fig. 1.3.2). Mean dimensions of 300 macroconidia was 4.54 ± 0.41 x 2.76 ± 0.29 μm, and the mean dimensions of 300 microconidia were 4.77 ± 0.83 x 1.38 ± 0.25 μm.

The highest growth rate of *F. kuroshium* occurred in cultures incubated in 25°C, with average colony diameter reaching 7.4 cm after 9 days of incubation (Fig. 1.4.2). This was in contrast to simultaneously incubated *F. euwallaceae* cultures showing the highest growth rate in 30°C with the average colony diameter of 7.0cm after 9 days of incubation. No overall significant effect was observed in interaction between *F. kuroshium* and *F. euwallaceae* by temperature. No growth occurred in cultures incubated in 5°C or 40°C. The highest growth rate of *G. kuroshium* and *G. euwallaceae* occurred in cultures incubated in 30°C, with the average colony diameter reaching 5.6 cm and 5.0 cm respectively after 14 days of incubation (Fig. 1.4.1). No overall significant effect was observed in interaction between *G. kuroshium* and *G. euwallaceae* by temperature. No growth occurred at temperatures of 5°C and 40°C for both *G. kuroshium* and *G. euwallaceae*.

Pathogenicity assay. - The avocado seedlings infected with *Fusarium euwallaceae, Fusarium kuroshium, Graphium euwallaceae* or *Graphium kuroshium* mostly did not show any outwardly visible symptoms 1 month after the fungal inoculations. Sugar (Perseitol) exudate resulting from mechanical damage of the phloem caused by the inoculation procedure was observed in most avocado seedlings used in the experiment, similarly to the observations of beetle-infested avocado trees in Israel by Mendel *et al.* (Mendel et al 2012, Liu et al 2002). Most plants treated with conidial suspension

injections showed xylem discoloration that radiated away from the inoculation points, whereas plants treated with sterile agar plugs showed xylem discoloration 0.5 cm~0.7 cm in length. *F. kuroshium*, *F. euwallaceae*, and *G. kuroshium* were recovered from all plants that were inoculated with the corresponding pathogens, and *G. euwallaceae* was recovered from 90% of all plants inoculated with the pathogen. No *Fusarium* or *Graphium* was recovered from plants treated with sterile agar plugs.

The mean lengths of lesions caused by *F. kuroshium* were found to be significantly different from those caused by *F. euwallaceae* ($P<0.0001$) (Fig. 1.5.1). There were no significant differences between the lesion sizes caused by different isolates of the same fungal species, and between the two experimental trials. 1 month after pathogen inoculation, avocado seedlings inoculated with *F. kuroshium* produced lesions with average length of 3.65 cm, and seedlings inoculated with *Fusarium euwallaceae* produced lesions with average length of 6.89 cm. Control seedlings treated with sterile agar plugs produced discolorations 0.56 cm long in average, which could be attributed to basal plant wound response.

The mean length of lesions in plants infected with *G. kuroshium* sp. nov. did not significantly differ from that of lesions in plants infected with *Graphium euwallaceae* (Fig. 1.5.2). No significant differences in mean lesion lengths were detected between the different isolates within the fungal species, as well as between the two trials. The mean length of lesions in plants infected with *Graphium kuroshium* or *Graphium euwallaceae* significantly differed from the mean length of xylem discoloration in plants treated with sterile agar plugs ($P<0.05$). Plants infected with *G. euwallaceae* showed lesions with the

average length of 4.91 cm, and those infected with *G. kuroshium*. showed lesions with the average length of 4.09 cm.

TAXONOMY

Fusarium kuroshium F. Na, A. Eskalen, sp. nov. FIGS. 1.1, 1.3.1

MycoBank xxxxxxxx

Typification: UNITED STATES. CALIFORNIA. El Cajon, San Diego County. Surface of *Euwallacea* sp. galleries in infested California sycamore (*Platanus racemosa*), 29 January 2014, *A. Eskalen,* (holotype NRRL xxxx = UCR3641).

Etymology: Derived from the common name of the vector ambrosia beetle, kuroshio shot hole borer (*Euwallacea* sp. #5) (O'Donnell *et al.* 2014)

Colonies grown on PDA in 25°C are initially white, colony center color changing to greyish red (10D4) with white margins in 14 day old cultures. Colony center color turns to greyish ruby (12E5) in 30 day old cultures. Reverse pigmentation in 14 day old cultures is brownish red (9C8) with orange margins (6A7), and reverse pigmentation turns to reddish brown (9E7) in 30 days. Sporodochial pigmentation is bluish green (25B8) to deep green (25D8). Colonies grown on SNA in 25°C are hyaline throughout, with more abundant aerial hyphae. Septate hyphae 3.75 ± 1.03 μm wide in SNA. Chlamydospores forms on hyphae and conidia, globose and smooth. Fusiform and clavate conidia with 0~5 septa produced on long aerial conidiophores. Septation increases as conidia mature, conidia dimensions are as follow. No septation: 9.63 ± 2.98 X 3.45 ± 0.81 μm average, 4.95-32.04 X 1.79-7.39 μm total range. 1-septate: 13.31 ± 3.23 X 4.42 ± 0.90 μm average, 6.70-23.44 X 2.36-7.06 μm total range. 2-septate: 24.68 ± 5.70 X

6.78 ± 1.12 µm average, 12.42-33.30 X 4.52-9.41 µm total range. 3-septate: 28.00 ± 3.99 X 7.18 ± 1.15 µm average, 19.45-35.69 X 5.11-10.13 µm total range. 4-septate: 34.87 ± 4.14 X 8.18 ± 1.12 µm average, 27.83-39.96 X 6.33-9.71 µm total range. 5-septate: 32.98 ± 0.66 X 7.67 ± 1.09 µm average, 32.51-33.44 X 6.90-8.44 µm total range.

Host Range: *Acer negundo, Persea americana, Platanus racemosa*

Fungal distribution: San Diego County, California, USA

Additional specimens examined: UNITED STATES. CALIFORNIA. El Cajon, San Diego County. Surface of *Euwallacea* sp. galleries in *Platanus racemosa,* 2014, F. Na, UCR3641, NRRL xxxx; UNITED STATES. CALIFORNIA. El Cajon, San Diego County. Surface of *Euwallacea* sp. galleries in *Platanus racemosa,* 2014, F. Na, UCR3644, NRRL xxxx; UNITED STATES. CALIFORNIA. Fallbrook, San Diego County. Head of *Euwallacea* sp. in infested *Persea americana,* 2015, F. Na, UCR3651, NRRL xxxx; UNITED STATES. CALIFORNIA. Bonsall, San Diego County. Surface of *Euwallacea* sp. galleries in *Persea americana,* 2015, F. Na, UCR3653, NRRL xxxx; UNITED STATES. CALIFORNIA. Bonsall, San Diego County. Surface of *Euwallacea* sp. galleries in *Persea americana,* 2015, F. Na, UCR3654, NRRL xxxx; UNITED STATES. CALIFORNIA. Bonsall, San Diego County. Head of *Euwallacea* sp. in infested *Persea americana,* 2015, F. Na, UCR3653, NRRL xxxx; UNITED STATES. CALIFORNIA. Bonsall, San Diego County. Head of *Euwallacea* sp. in infested *Persea americana,* 2015, F. Na, UCR3657, NRRL xxxx; UNITED STATES. CALIFORNIA. Bonsall, San Diego County. Head of *Euwallacea* sp. in infested *Persea americana,* 2015, F. Na, UCR3659, NRRL xxxx; UNITED STATES. CALIFORNIA. Bonsall, San Diego

County. Head of *Euwallacea* sp. in infested *Persea americana,* 2015, F. Na, UCR3660, NRRL xxxx; UNITED STATES. CALIFORNIA. Escondido, San Diego County. Surface of *Euwallacea* sp. galleries in *Persea americana,* 2015, F. Na, UCR3661, NRRL xxxx; UNITED STATES. CALIFORNIA. Escondido, San Diego County. Surface of *Euwallacea* sp. galleries in *Persea americana,* 2015, F. Na, UCR3662, NRRL xxxx.

Comments: *F. kuroshium* resembles the related ambrosial *Fusarium* species *F. euwallaceae* in various morphological features. *F. kuroshium* has been observed to differ from *F. euwallaceae* only in colony pigmentation, which is lighter in color. 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 (Shown as *Fusarium* sp. I Taiwan in phylogenetic tree) compared to *F. euwallaceae*.

Graphium kuroshium F. Na, A. Eskalen, sp. nov. FIGS. 1.2, 1.3.2

MycoBank xxxxxxxx

Typification: UNITED STATES. CALIFORNIA. Fallbrook, San Diego County. Surface of *Euwallacea* sp. galleries in infested avocado (*Persea americana*), 20 November 2015, *A. Eskalen,* (holotype NRRL xxxx).

Etymology: Derived from the common name of the vector ambrosia beetle, kuroshio shot hole borer (*Euwallacea* sp. #5) (O'Donnell *et al.* 2014)

Colonies grown on PDA in 25°C are initially white, colony center color changing to dark green (25F4) with yellowish white margins (3A2-3B2) in 14 day old cultures. Colony center color darkens over time. Fruiting body pigmentation is black, and can be seen

emerging from wood surface within established galleries. Aerial hyphae are hyaline on PDA, colonies grown on SNA in are flat. Colonies within cultures grown 10°C, 15°C, and 35°C show reduced yellowish white (3A2) colonies with irregular borders and mucoid texture, cultures grown in 20°C, 25°C and 30°C show larger colonies possessing regular borders. Two morphologically distinct conidia are produced from synnemata. Synnemata consist of packed parallel hyphae 1.36 ± 0.28 μm in width each. Synnemata are 9.88 ± 2.24 μm wide at the top, 6.12 ± 1.98 μm wide at the bottom. Abundant hyphae rhizoid in shape present in the bottom of synnemata. Macroconidia of dimensions 4.54 ± 0.41 X 2.76 ± 0.29 μm (3.41-5.6 X 1.84-3.58 μm total range) show annelidic development in conidiogenous structures located in the tops of synnemata, and Microconidia of dimensions 4.77 ± 0.83 X 1.38 ± 0.25 μm (2.99-7.24 X 0.77-2.30 μm total range) develop from conidiogenous structures located in the rhizoid-like bottom portions of synnemata. Macroconidia are aseptate, thick-walled and symmetrical ellipsoid in shape, microconidia are aseptate, cylindrical in shape with truncated ends.

Host Range: *Acer negundo, Persea americana, Platanus racemosa*

Fungal distribution: San Diego County, California, USA

Additional specimens examined: UNITED STATES. CALIFORNIA. Fallbrook, San Diego County. Surface of *Euwallacea* sp. galleries in *Persea americana,* 2015, F. Na, UCR4593, NRRL xxxx; UNITED STATES. CALIFORNIA. Fallbrook, San Diego County. Surface of *Euwallacea* sp. galleries in *Persea americana,* 2015, F. Na, UCR4594, NRRL xxxx; UNITED STATES. CALIFORNIA. Bonsall, San Diego County. Surface of *Euwallacea* sp. galleries in *Persea americana,* 2015, F. Na, UCR4606, NRRL

xxxx; UNITED STATES. CALIFORNIA. Bonsall, San Diego County. Surface of *Euwallacea* sp. galleries in *Persea americana,* 2015, F. Na, UCR4607, NRRL xxxx; UNITED STATES. CALIFORNIA. Bonsall, San Diego County. Head of *Euwallacea* sp. in infested *Persea americana,* 2015, F. Na, UCR4608, NRRL xxxx; UNITED STATES. CALIFORNIA. Bonsall, San Diego County. Head of *Euwallacea* sp. in infested *Persea americana,* 2015, F. Na, UCR4609, NRRL xxxx; UNITED STATES. CALIFORNIA. Escondido, San Diego County. Surface of *Euwallacea* sp. galleries in *Persea americana,* 2015, F. Na, UCR4616, NRRL xxxx; UNITED STATES. CALIFORNIA. Escondido, San Diego County. Surface of *Euwallacea* sp. galleries in *Persea americana,* 2015, F. Na, UCR4617, NRRL xxxx; UNITED STATES. CALIFORNIA. Escondido, San Diego County. Head of *Euwallacea* sp. in infested *Persea americana,* 2015, F. Na, UCR4618, NRRL xxxx; UNITED STATES. CALIFORNIA. Escondido, San Diego County. Head of *Euwallacea* sp. in infested *Persea americana,* 2015, F. Na, UCR4622, NRRL xxxx;

Comments: *G. kuroshium* colonies are morphologically identical to *G. euwallaceae*, ambrosial *Graphium* species associated with polyphagous shot hole borer (*Euwallacea* sp. #1) (O'Donnell *et al.* 2014), in culture. Conidial dimensions of macroconidia produced by *G. kuroshium* slightly differ from those produced by *G. euwallaceae*, as the average length of macroconidia produced by *G. kuroshium* was lower than the minimum length observed in macroconidia produced by *G. euwallaceae* (Lynch *et al.* 2016). Microconidia 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.* The results of the phylogenetic analyses are

consistent with the finding by O'Donnell *et al.* (2014) and Cooperband *et al.* (2016), which showed that the vector ambrosia beetle species of *G. kuroshium* (*Euwallacea* sp. #5, O'Donnell *et al.* 2014) are genetically distinct from the vector beetle species of *G. euwallaceae* (*Euwallacea* sp. #1, O'Donnell *et al.* 2014).

DISCUSSION

The results from this study show that the symbiotic fungal species associated with Kuroshio shot hole borer are related to but distinct from those associated with polyphagous shot hole borer. *Fusarium kuroshium* has been observed to differ from *Fusarium euwallaceae* in morphology, genetic composition, and symptom severity, whereas the genetic composition was the most compelling evidence that *Graphium kuroshium*. differs from *Graphium euwallaceae*.

During the fungal isolate collection, we noted that *Paracremonium pembeum*, a PSHB-associated symbiotic fungal species, has never been isolated from KSHB or its galleries. This finding was notable because *P. pembeum* has previously been isolated along with *Fusarium euwallaceae* and *Graphium euwallaceae* from both the heads of the PSHB beetles and their galleries (Lynch *et al.*, 2016). The absence of *P. pembeum* or any related species in KSHB mycangia or galleries presented us with a question: Does the absence of *P. pembeum* affect the fitness of the KSHB beetles compared to PSHB, and what role does *P. pembeum* perform in its association with PSHB? The presence of *F. kuroshium* and *G. kuroshium* in KSHB mycangia and galleries may be important to the fitness of KSHB – *Fusarium* and *Graphium* have historically been known to be

commonly associated with the Scolytine tribe Xyleborini, which includes the genus *Euwallacea*. *Fusarium* has been found as a secondary associate of Xyleborini spp. (Kostovcik *et al.,* 2015), and the primary fungal associate of most ambrosia beetle species belonging in the genus *Euwallacea* (Kasson *et al.,* 2013). The genus *Graphium* belongs to the order Microascales, and Microascales fungal species have been found as associates of many species of ambrosia beetles and bark beetles belonging to the tribe Xyleborini. Microascales species often serve as the primary fungal associate of many Xyleborini, such as the beetles belonging to the genus *Xyleborus*. (Alamouti *et al.,* 2009; Beaver 1989; Harrington *et al.,* 2014). While fungal species belonging to the genus *Acremonium* such as the *P. pembeum* are not quite as commonly found as an associate of ambrosia beetles, they nevertheless have been found in association with ambrosia beetles in several occasions; *Cephalosporium pallidum* (now classified under *Acremonium*) has been found associated with the ambrosia beetle *Xyleborus affinis* (Verrall 1943; Biedermann *et al.,* 2009), *Cephalosporium* sp. (along with *Fusarium solani* and *Graphium* sp.) have been found associated with the ambrosia beetle *Xyleborus ferrugineus* (Baker and Norris, 1968), and most recently, *P. pembeum* has been found to be associated with PSHB (Lynch *et al.,* 2016). The absence of *P. pembeum* in the mycangia of KSHB may simply be because the fungus is a secondary fungal associate of PSHB. Ambrosia beetles generally favor a single fungal associate as the primary fungal associate (Mueller *et al.,* 2005; Gebhardt *et al.,* 2004), and *F. euwallaceae* and *F. kuroshium* may respectively be those of PSHB and KSHB since the *Fusarium* species are the most abundantly found in the beetles and their galleries. Secondary fungal associates may have some unknown

effects in beetle development, even if the fungi are not found in the mycangia of the beetles (Mueller *et al.,* 2005), and it is possible that *P. pembeum* was lost as a secondary fungal associate somewhere in the evolutionary timeline of KSHB. From a glance, KSHB fitness does not seem to be affected by the apparent lack of *P. pembeum* – the beetle has successfully colonized a large portion of the county of San Diego, and it is currently moving northward towards Orange County, where PSHB is known to be present. A proper study in the future may be necessary to assess the effects of each fungal associate on the ambrosia beetles.

We have also noted during the fungal isolate collection that the plates spread with suspensions containing beetle head or gallery surface scrapings often contained only *F. kuroshium* and *G. kuroshium*, with very little to no contaminants. This may be due to fungal garden maintenance by the beetles, in which the beetles preserve the fungal gardens from contamination by actively maintaining the gardens (Batra 1967; Beaver 1989). This ensures the growth of desirable ambrosia fungi, and the inhibition of undesirable parasites or pathogens. This type of interaction has been studied in the Southern pine beetles, *Dendroctonus frontalis,* which is known to maintain the primary fungal associate *Entomocorticium* sp. by inhibiting the secondary fungal associate *Ophiostoma minus* (Klepzig and Wilkens 1997; Hofstetter *et al.,* 2006). While *O. minus* acts to suppress host defense response in the early stages of the beetle attacks, *O. minus* can outcompete *Entomocorticium* sp. in gallery, making it an undesirable fungus to be present in galleries. The beetle is known to selectively inhibit *O. minus* by utilizing a mutualistic bacterial species. Ambrosia beetles have also been observed to harbor

bacterial or yeast communities in mycangia, and thus it is possible that the PSHB and KSHB maintain their fungal gardens using bacterial or yeast symbionts (Hulcr *et al.*, 2012; Kajimura and Hijii 1992). The suppression of fungal garden maintenance by inhibition of bacterial or yeast symbionts may be a method that can be utilized additionally to control the beetles, and future studies to elucidate the PSHB/KSHB fungal garden maintenance process may be an interesting direction for future research.

Phylogenetic analysis of *Fusarium kuroshium* revealed its evolutionary relationship with *Fusarium euwallaceae* and *Fusarium* sp. I. The placement of two *Fusarium* sp. I isolates originating from Taiwan as a subclade within the clade containing *F. kuroshium* suggests that the *F. kuroshium* obtained from the KSHB beetles and their galleries are related closer to *Fusarium* spp. that occur natively in Taiwan than to *Fusarium euwallaceae*. This finding is consistent with the current hypothesis that two separate invasion events lead to the introduction of *F. euwallaceae* and *F. kuroshium* to Los Angeles County and San Diego County, respectively, and it also naturally suggests that the beetle vector of *F. kuroshium* is descended from *Euwallacea* spp. that occur in Taiwan. Phylogenetic analysis of *Graphium kuroshium* also revealed that the *Graphium* species obtained from KSHB beetles and their galleries were genetically distinct from *Graphium euwallaceae* and other *Graphium* species observed during this study. This finding further supports the hypothesis that KSHB invasion originated from a location geographically separated from PSHB's original habitat, although we were unable to confirm *G. kuroshium*'s relationship to a *Graphium* species associated with *Euwallacea* spp. in Taiwan. The existence of a *Graphium* associate of *Euwallacea* spp. in Taiwan is

currently unconfirmed. As Taiwan is host to 63 species belonging to the tribe Xyleborini, 6 of which are known to be of the genus *Euwallacea* (Beaver and Liu 2010), Taiwan as the native range of the ancestor of KSHB is certainly a possibility. While the placement of *Fusarium* sp. I isolates in the clade with *Fusarium kuroshium* supports the hypothesis that *F. kuroshium* is a descendent of *Fusarium* sp. I, the remaining 10 *Fusarium* isolates from Taiwan formed several clades of their own that did not cluster with *Fusarium euwallaceae* of PSHB or *Fusarium ambrosium* of *E. fornicatus.* This suggests genetic diversity within the *Fusarium* sp. that occur in Taiwan, which differs from the lack of such diversity within the *F. kuroshium*. As teleomorphs of *F. euwallaceae* and *F. kuroshium* have not been observed, the genetic variation found in the Taiwan isolates is unusual; *Fusarium euwallaceae* isolates obtained from Los Angeles County and Israel have not been found to differ genetically, despite the large geographical separation of the two niches (Freeman *et al.*, 2013). The lack of genetic variation in *F. kuroshium* may be attributable to the highly specialized beetle-symbiotic fungus's reproductive success as an associate of the highly successful invasive ambrosia beetle, though this hypothesis alone may not be sufficient to explain the discrepancy in genetic diversity. The genetic variation found could simply be due to accidental sampling a mix of different *Euwallacea* spp. in Taiwan. 6 *Euwallacea* species are known to occur in Taiwan (Beaver and Liu 2010), and many of the *Euwallacea* species share similar morphological characteristics (Smith and Hulcr 2015), likely due to their highly evolved lifestyles as xylem-boring insects. The genetic variation may also be due to mixing of several species of ambrosia fungi within the beetle galleries. The fungal associates of the 6 ambrosia beetle species in

Taiwan are not yet well understood, but fungal stealing among ambrosia and bark beetles is known to have evolved independently multiple times (Hulcr and Cognato 2010). If any fungal stealing among the 6 species of beetles has occurred in Taiwan, it may be possible that the *Fusarium* associates of the beetles have formed hybrids during this process, allowing for genetic diversity in the fungi. Lastly, it could simply be that *Fusarium kuroshium* and its vectors have not been in this new niche for long enough time for it to show variation. In order to better elucidate the geographical origins of the ancestors of *F. euwallaceae* and *F. kuroshium*, it may be necessary to conduct additional phylogenetic analyses involving more *Fusarium* isolates from Taiwan and other locations that harbor *Euwallacea* spp.

Based on the observations during the pathogenicity trials, it is likely that *Fusarium kuroshium* is a weak plant pathogenic fungal associate of KSHB. The symptom severity of *F. kuroshium* observed during the pathogenicity trial was significantly less severe compared to *F. euwallaceae,* with the mean lesion size only reaching about half of *F. euwallaceae*'s. *G. kuroshium,* on the other hand, was found to cause symptoms that were similar to those of *G. euwallaceae* in severity. In relative terms, the symptom severity of *G. kuroshium* and *G. euwallaceae* was in between that of *F. kuroshium* and *F. euwallaceae*, showing that all four species of beetle-associated fungi were essentially weak pathogens. The low symptom severity from a single point of inoculation does not necessarily equate to lower risk, however; when introduced into the xylem *en masse* by a large number of invading beetles, even weak pathogenic fungi such as *F. kuroshium* and *G. kuroshium* can quickly occlude the xylem vessels of a host plant. This mass attack

behavior of PSHB and KSHB has been observed in Israel and California (Eskalen *et al.*, 2013; Mendell *et al.,* 2012), and this behavior has been observed in other ambrosia beetle species such as *Euwallacea destruen* Blandford and *Coptoborus ochromactonus* as well (Browne, 1958; Stilwell *et al.*, 2014; Smith and Hulcr 2015). It is currently not clear whether the reduced symptom severity in *F. kuroshium* has any effect in KSHB beetle fitness. While there is not enough data to directly compare the fitness of PSHB and KSHB, it is clear that both ambrosia beetle species were successful in colonizing their niches in California. It is possible to hypothesize that the reduced symptom severity in *Fusarium kuroshium* is actually an adaptation that allows the KSHB beetles to colonize while eliciting lower plant defense response from host plants. While host defense suppression by beetle-associated fungal species has been observed in bark beetles, such as the secondary fungal associate *Ophiostoma minus* of the bark beetle *Dendroctonus frontalis* (Klepzig and Hofstetter, 2011) and the *Ophiostoma clavigerum* of the mountain pine beetle *Dendroctonus ponderosae* Hopkins (Diguistini *et al.,* 2007), such adaptation has not yet been observed in ambrosia beetles.

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Table 1.1. Isolates used in this study

Isolate ID	Local ID	Location	Host	Identity
UCR3641	G66	El Cajon, CA	*Platanus racemosa*	*F. kuroshium*
UCR3644	G69	El Cajon, CA	*Platanus racemosa*	*F. kuroshium*
UCR3651	TF4	Fallbrook, CA	Head of *Euwallacea* sp.	*F. kuroshium*
UCR3653	WPF1	Bonsall, CA	*Persea americana* cv. Hass	*F. kuroshium*
UCR3654	WPF2	Bonsall, CA	*Persea americana* cv. Hass	*F. kuroshium*
UCR3657	PF1	Bonsall, CA	Head of *Euwallacea* sp.	*F. kuroshium*
UCR3659	PF4	Bonsall, CA	Head of *Euwallacea* sp.	*F. kuroshium*
UCR3660	PF5	Bonsall, CA	Head of *Euwallacea* sp.	*F. kuroshium*
UCR3661	WGF1	Escondido, CA	*Persea americana* cv. Hass	*F. kuroshium*
UCR3662	WGF2	Escondido, CA	*Persea americana* cv. Hass	*F. kuroshium*
UCR4672	EF4	Taichung, Taiwan	Head of *Euwallacea fornicatus*	*Fusarium* sp.
UCR4673	EF6	Taichung, Taiwan	Head of *Euwallacea fornicatus*	*Fusarium* sp.
UCR4674	EF8	Taichung, Taiwan	Head of *Euwallacea fornicatus*	*Fusarium* sp.
UCR4675	EF9	Taichung, Taiwan	Head of *Euwallacea fornicatus*	*Fusarium* sp.
UCR4676	EF10	Taichung, Taiwan	Head of *Euwallacea fornicatus*	*Fusarium* sp.
UCR4677	EF11	Taichung, Taiwan	Head of *Euwallacea fornicatus*	*Fusarium* sp.
UCR4678	EF12	Taichung, Taiwan	Head of *Euwallacea fornicatus*	*Fusarium* sp.
UCR4679	BtF01	Taichung, Taiwan	Head of *Euwallacea fornicatus*	*Fusarium* sp.
UCR4680	Fe01	Taichung, Taiwan	Head of *Euwallacea fornicatus*	*Fusarium* sp.
UCR4681	Fuw01	Taichung, Taiwan	Head of *Euwallacea fornicatus*	*Fusarium* sp.
UCR4593	WTG1	Fallbrook, CA	*Persea americana* cv. Hass	*G. kuroshium*
UCR4594	WTG2	Fallbrook, CA	*Persea americana* cv. Hass	*G. kuroshium*
UCR4606	WPG4	Bonsall, CA	*Persea americana* cv. Hass	*G. kuroshium*
UCR4607	WPG5	Bonsall, CA	*Persea americana* cv. Hass	*G. kuroshium*
UCR4608	PG1	Bonsall, CA	Head of *Euwallacea* sp.	*G. kuroshium*
UCR4609	PG2	Bonsall, CA	Head of *Euwallacea* sp.	*G. kuroshium*
UCR4616	WGG4	Escondido, CA	*Persea americana* cv. Hass	*G. kuroshium*
UCR4617	WGG5	Escondido, CA	*Persea americana* cv. Hass	*G. kuroshium*
UCR4618	GG1	Escondido, CA	Head of *Euwallacea* sp.	*G. kuroshium*
UCR4622	GG5	Escondido, CA	Head of *Euwallacea* sp.	*G. kuroshium*

Table 1.2. Average dimensions of *Fusarium kuroshium* conidia by media used and septation levels

	PDA		SNA	
Septation	Avg Length	Avg Width	Avg Length	Avg Width
0	9.63	3.45	9.47	3.39
1	13.31	4.42	13.16	4.34
2	24.68	6.78	26.05	7.34
3	28.00	7.18	28.24	7.52
4	34.87	8.18	36.39	9.11
5	32.98	7.67	N/A	N/A

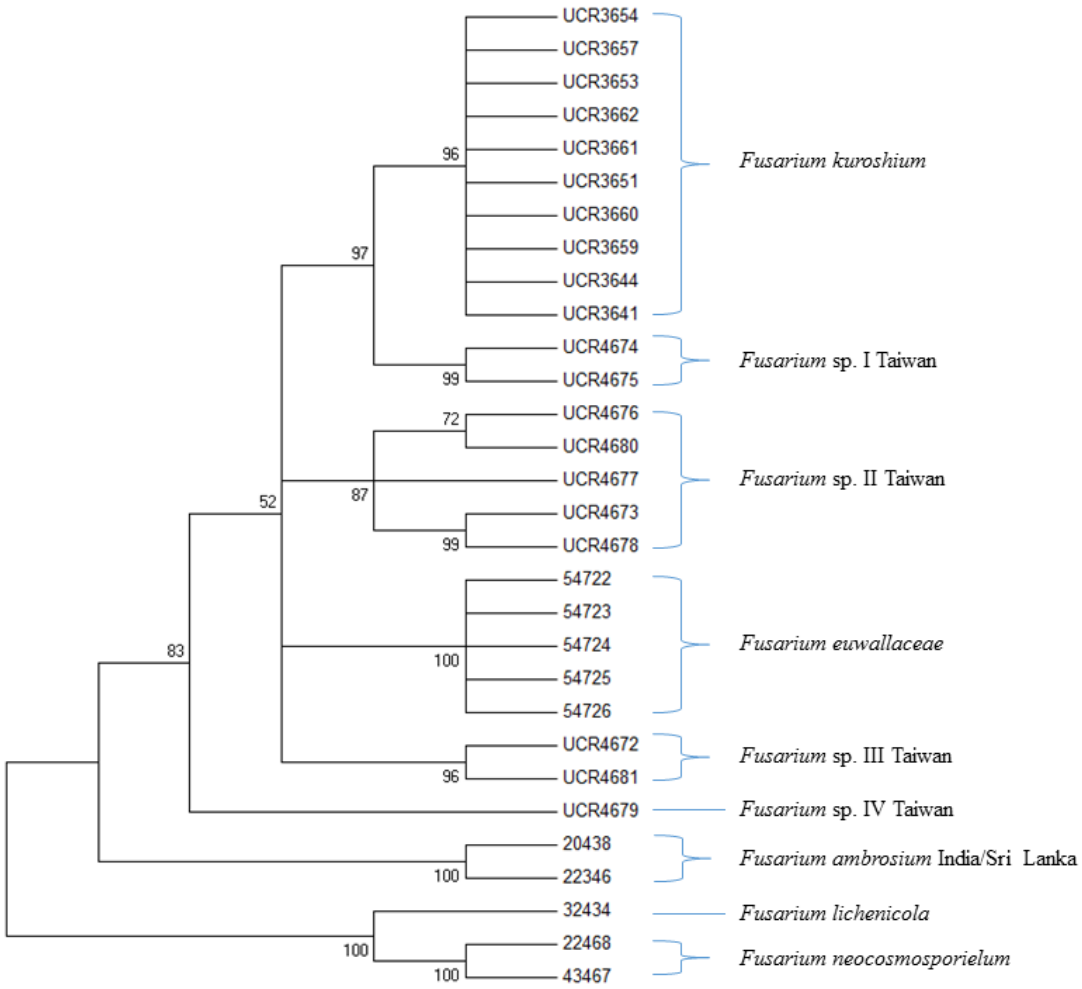

Figure 1.1. Multilocus phylogenetic analysis of *Fusarium* spp. conducted with four genes: ribosomal internal transcribed spacer (ITS), elongation factor 1-α (EF1-α), DNA-directed RNA polymerase II largest subunit (RPB1), DNA-directed RNA polymerase II second largest subunit (RPB2). Diagram was constructed using maximum likelihood method based on the Tamura-Nei model.

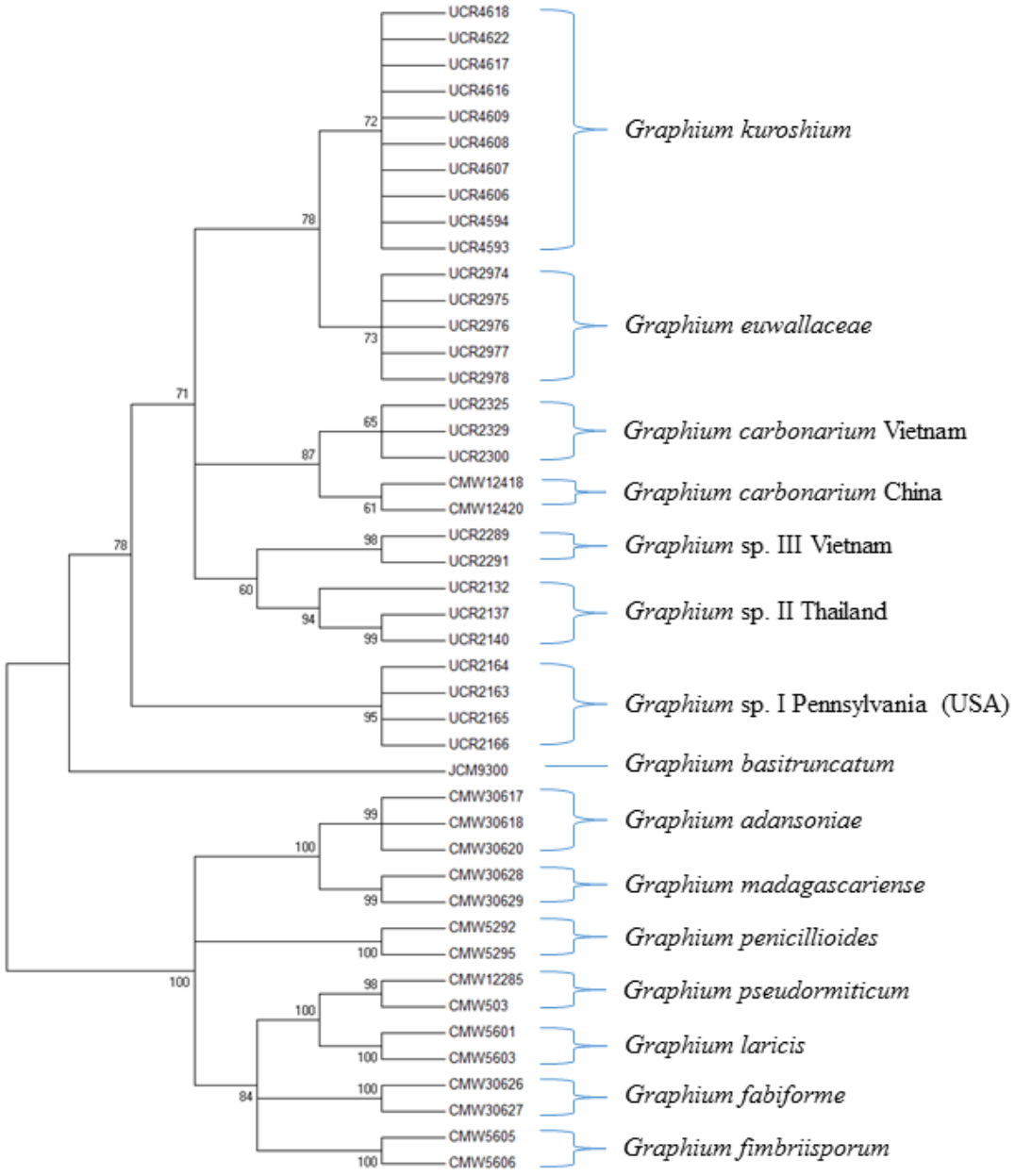

Figure 1.2. Multilocus phylogenetic analysis of *Fusarium* spp. conducted with four genes: ribosomal internal transcribed spacer (ITS), elongation factor 1-α (EF1-α), DNA-directed RNA polymerase II largest subunit (RPB1), DNA-directed RNA polymerase II second largest subunit (RPB2). Diagram was constructed using maximum likelihood method based on the Tamura-Nei model.

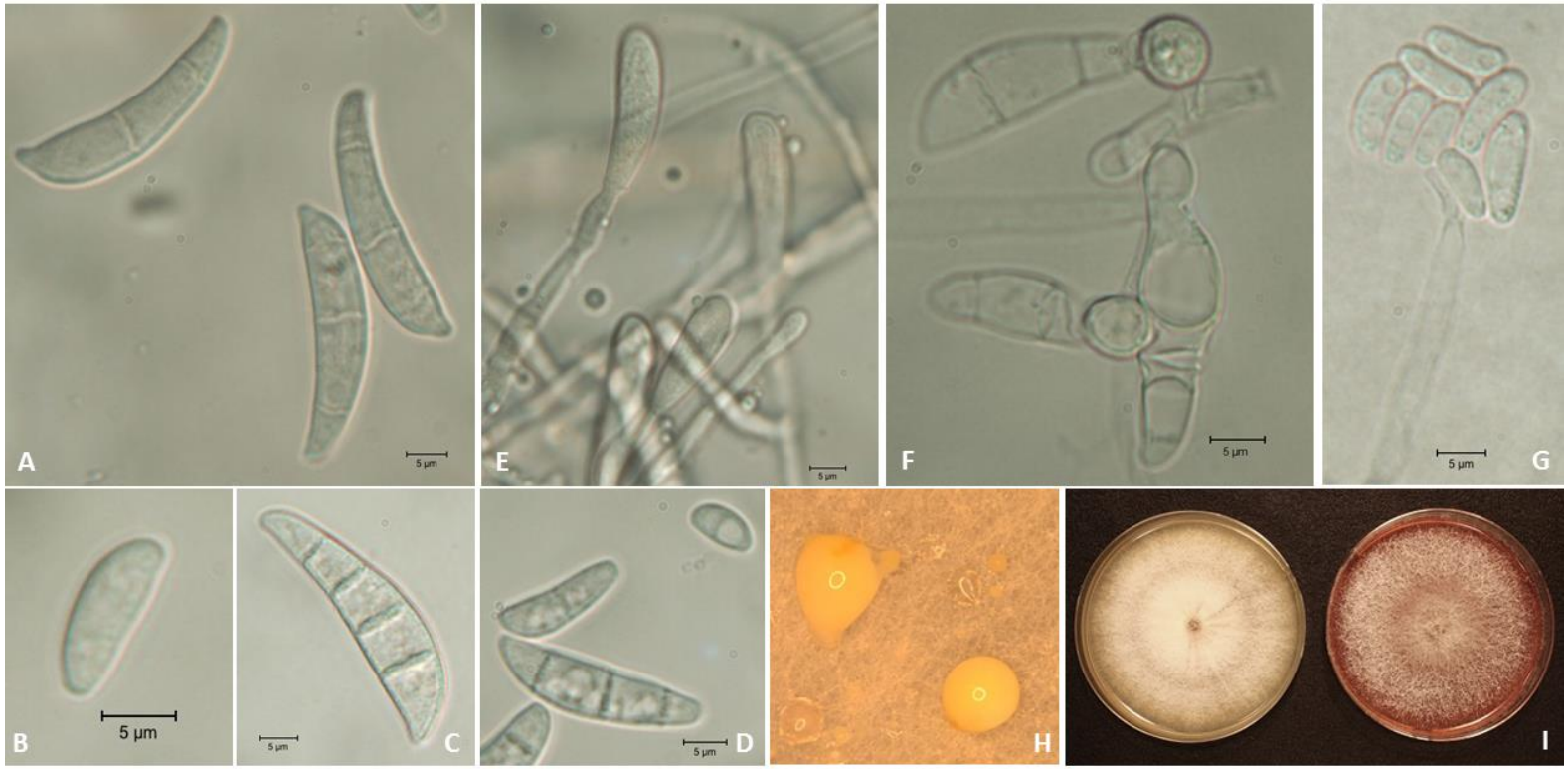

Figure 1.3.1. Morphology of *F. kuroshium* 14-day old cultures grown on PDA (A, C, E, F, H) or SNA (B, D, G). Conidia are multiseptate, and fusiform-clavate in shape. Fusiform 2-septate conidia (A) aseptate conidium in SNA (B) 4-septate conidium (C), 1~3 septate conidia in SNA (D) are shown. (E) shows conidiogenous cells on conidiophore, (F) Chlamydospores on conidia. (G) a mass of conidia on conidiophore. (H) Sporodochia on PDA after 4 wks. (I) *Fusarium euwallaceae* (on the right) and *F. kuroshium* (on the left) cultures in PDA to show difference in colony pigmentation.

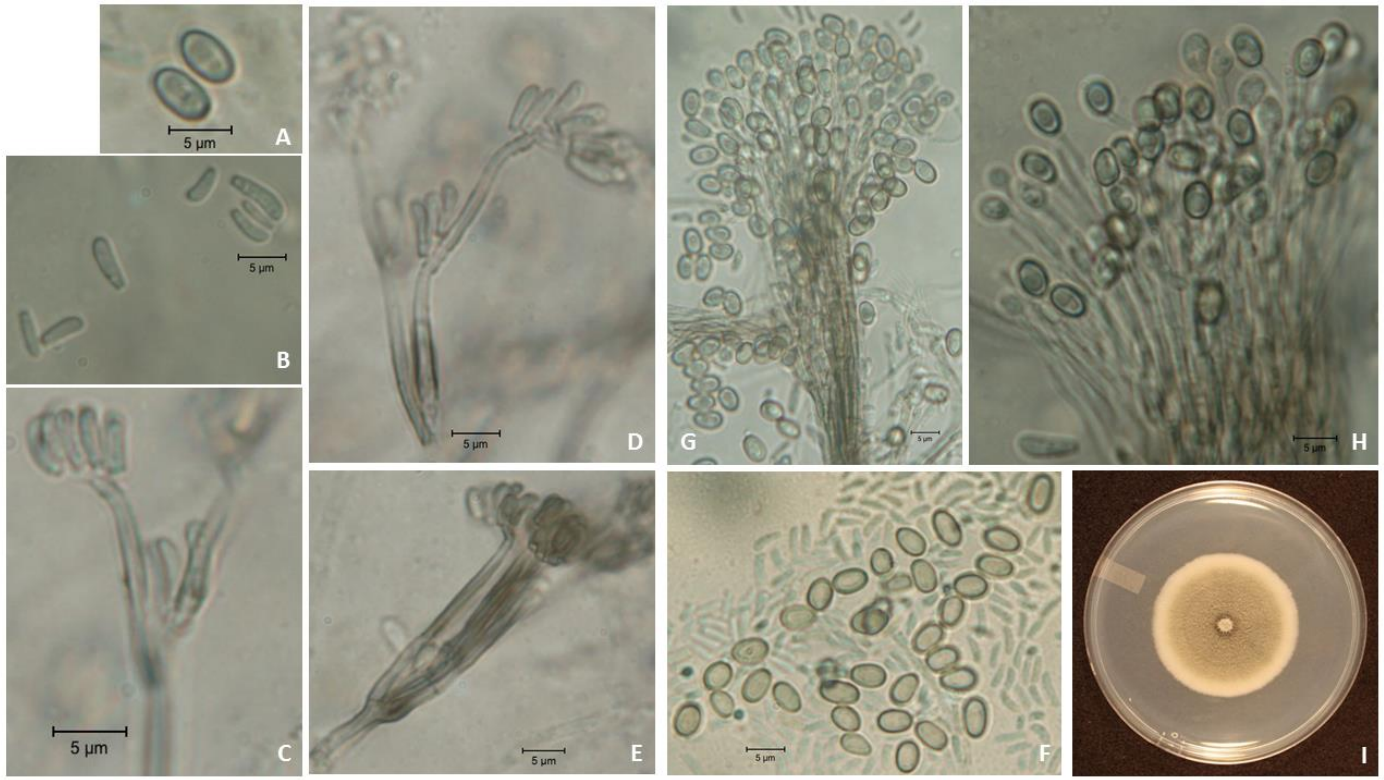

Figure 1.3.2. Morphology of *Graphium* sp. 14-day old cultures grown on oat meal agar (A~H) and PDA (I). *Graphium* sp. produces 2 morphologically distinct conidia: macroconidia are aseptate, thick-walled and symmetrical ellipsoid in shape (A), microconidia are aseptate, cylindrical in shape with truncated ends (B). Mass of microconidia on conidiophore (C~E), macro and microconidia mixed, microconidia are stained with lactophenol cotton blue (F), synnema bearing macroconidia (G~H), 14-day old *Graphium* sp. culture on PDA (I)

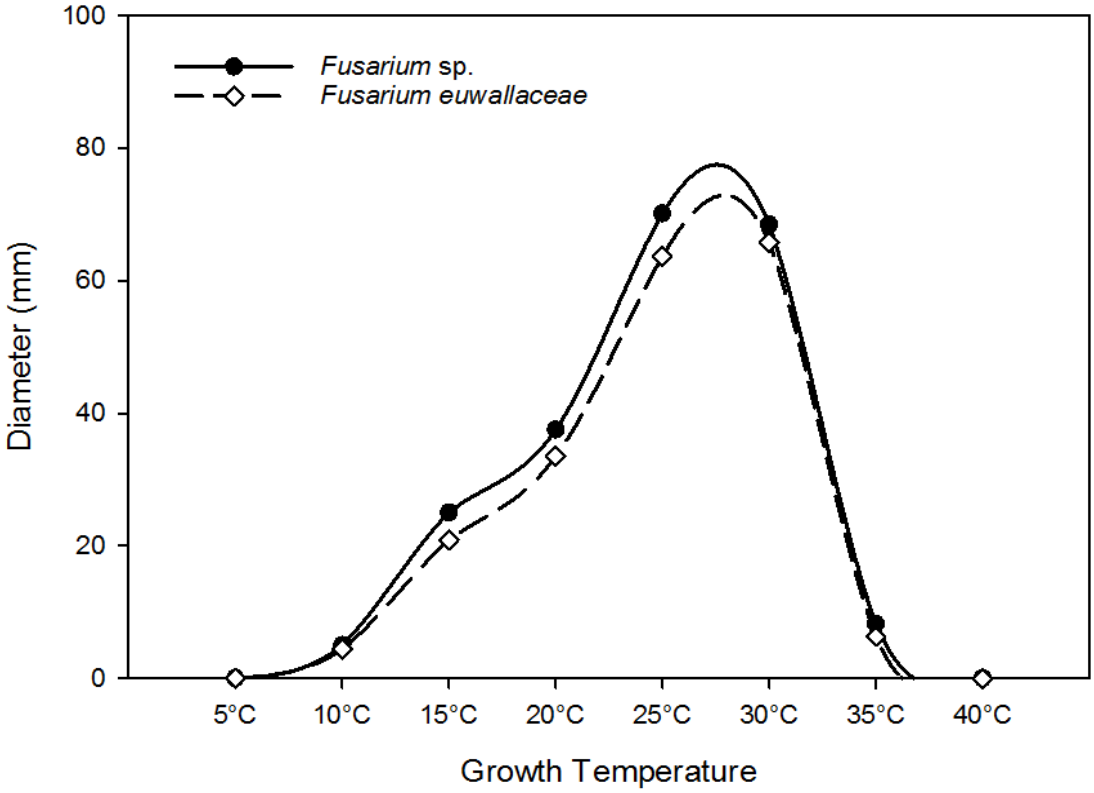

Figure 1.4.1. Radial growth of *F. kuroshium* and *Fusarium euwallaceae.* Cultures were prepared by placing agar plugs containing *F. kuroshium* or *Fusarium euwallaceae* on PDA, and cultures were incubated in temperatures shown in the x axis for 9 days. Diameters of the resulting colonies were measured after 9 days of incubation. *F. kuroshium* showed slightly higher growth rate compared to *Fusarium euwallaceae* across all temperatures tested. The highest growth rate occurred at 30°C for *Fusarium euwallaceae,* whereas the highest growth rate for *F. kuroshium* occurred at 25°C. No growth occurred in 5°C and 40°C for both isolates.

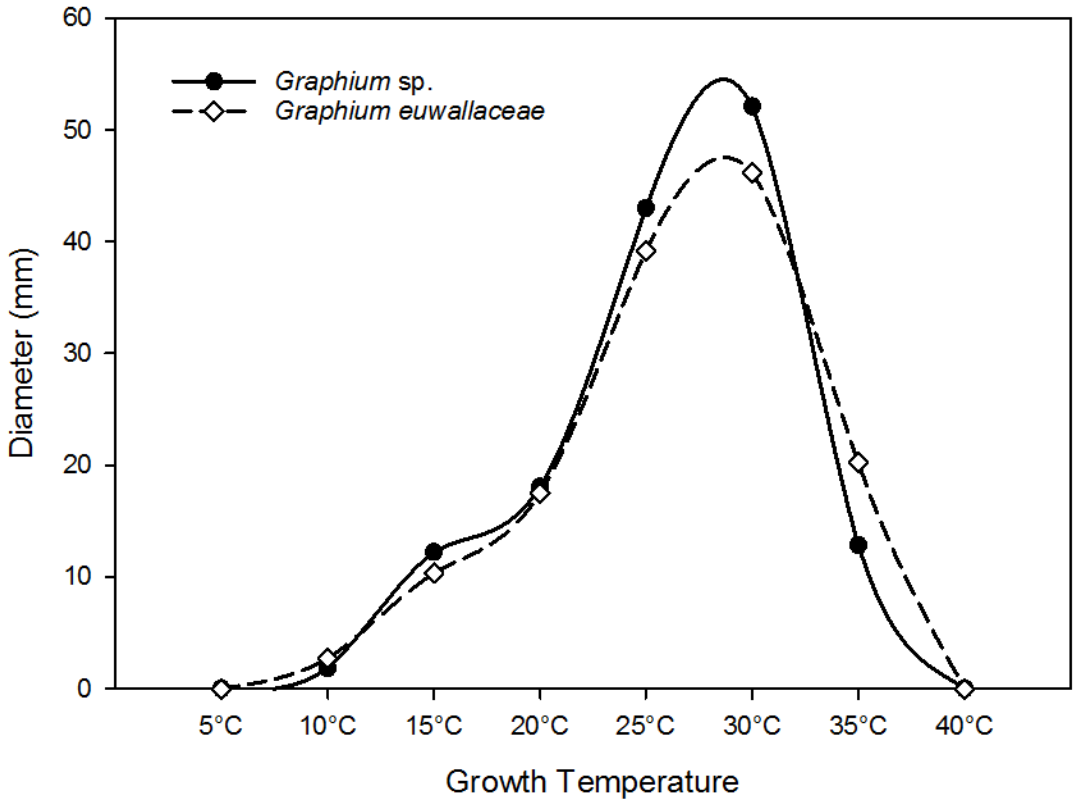

Figure 1.4.2. Radial growth of *Graphium* sp. and *Graphium euwallaceae.* Cultures were prepared by placing agar plugs containing *Graphium* sp. or *Graphium euwallaceae* on PDA, and cultures were incubated in temperatures shown in the x axis for 14 days. Diameters of the resulting colonies were measured after 14 days of incubation. *Graphium* sp. showed slightly higher growth rate in 25°C and 30°C, but showed reduced colony size at 35°C compared to *Graphium euwallaceae.* No growth occurred at 5°C and 40°C for both isolates.

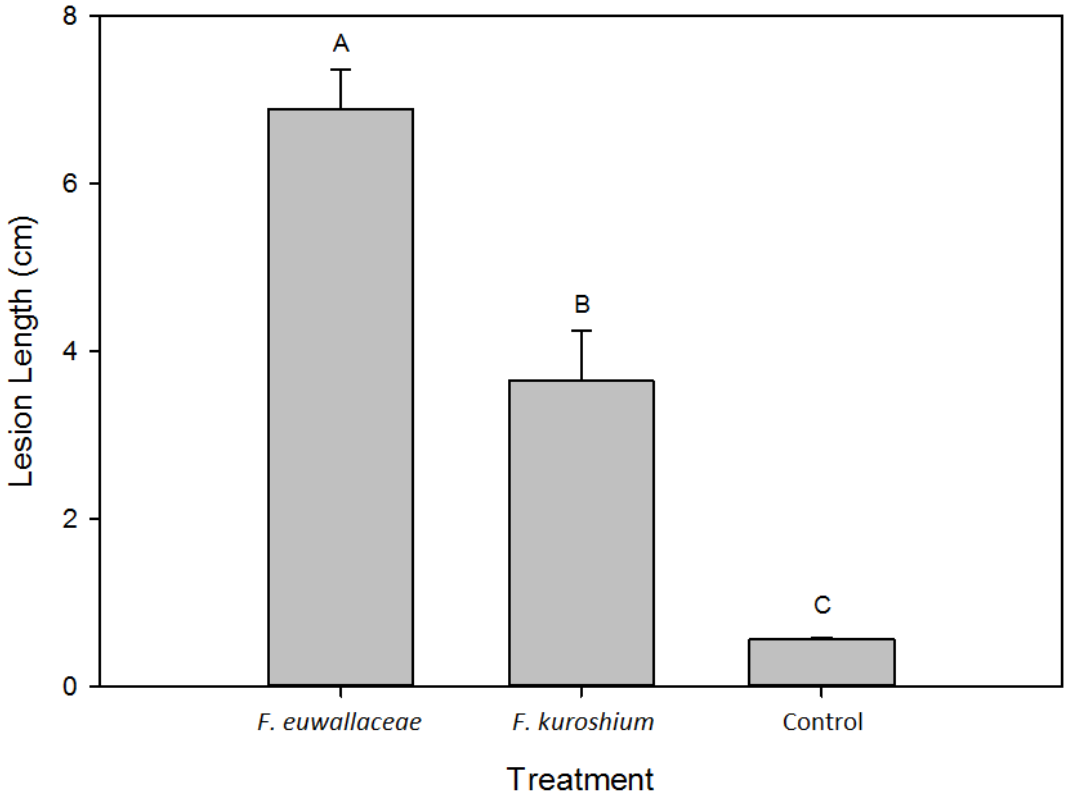

Figure 1.5.1. Length of xylem discoloration caused by *F. euwallaceae* and *F. kuroshium* in 2-year old *Persea americana* seedlings. Seedlings were inoculated with agar plugs containing *F. euwallaceae* or *F. kuroshium* mycelia and were incubated for 30 days prior to measuring the discoloration on xylem. Control plants were treated with sterile agar plugs, and produced discolorations ~0.5 cm in length. Statistical analysis was performed using Tukey's honest significant difference (HSD) test at $\alpha = 0.05$. Levels connected by the same letter are not significantly different.

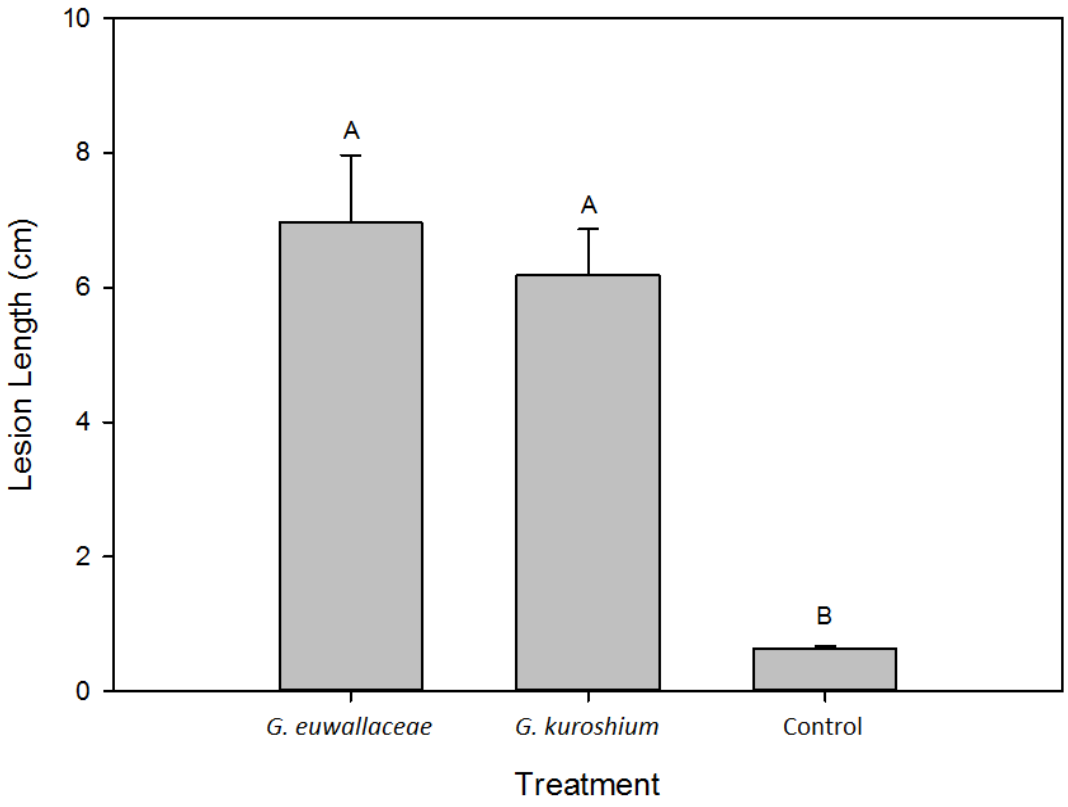

Figure 1.5.2. Length of xylem discoloration caused by *Graphium* sp. and *Graphium euwallaceae* in 2-year old *Persea americana* seedlings. Seedlings were inoculated with agar plugs containing *G. euwallaceae* or *G. kuroshium* mycelia and were incubated for 30 days prior to measuring the discoloration on xylem. Control plants were treated with sterile agar plugs, and produced discolorations ~0.5 cm in length. Statistical analysis was performed using Tukey's honest significant difference (HSD) test at $\alpha = 0.05$. Levels connected by the same letter are not significantly different.

Chapter 2:

Evaluation of the Use of Bacterial Endophytes of Avocado and California Sycamore as Biological Control to Inhibit the Fungal Associates of the Invasive Ambrosia Beetle Species (*Euwallacea* spp.) in California

Abstract:

The invasive ambrosia beetle species polyphagous shot hole borer (*Euwallacea* sp. I, "PSHB") and kuroshio shot hole borer (*Euwallacea* sp. II, "KSHB") are known to have an obligate association with several plant-pathogenic fungal species, including *Fusarium euwallaceae* in PSHB and *Fusarium kuroshium* in KSHB. Both beetle species are highly dependent on the successes of these fungal associates in colonizing the wood tissues of their hosts, because the ambrosial *Fusarium* species serve as the beetles' sole source of nutrients. To determine whether endophytic bacteria of the host plant species can be used as biological control agents to inhibit the growth of *Fusarium* ambrosial fungi of PSHB and KSHB, *in vitro* screen and *in planta* greenhouse injection assays were conducted. Endophytic bacterial isolates were obtained from the wood tissues of common beetle reproductive hosts, avocado (*Persea americana*) and California sycamore (*Platanus racemosa*), and were identified using molecular identification methods. These bacteria were simultaneously cultured with *F. euwallaceae* in order to screen for endophytic bacteria with the ability to inhibit the *Fusarium* ambrosial fungi. 15 bacterial isolates were found to inhibit *F. euwallaceae* during the screen, and all 15 were identified as belonging to the *Bacillus subtilis* species complex. The inhibition efficacies of the

bacterial endophytes were calculated for both *Fusarium* associates of PSHB and KSHB as percent inhibition, and four bacterial isolates that showed the highest percent inhibition were chosen to be used as injection treatments in the greenhouse *in planta* study. In greenhouse, stems of avocado and California sycamore were bacterized using the four chosen *Bacillus* sp. isolates then challenged with *F. euwallaceae* or *F. kuroshium.* The sizes of fungal lesions resulting from the *Fusarium* infection were measured and compared. The results showed that some endophytic bacteria had significant effects on the sizes of lesions. However, the *Bacillus* sp. isolate that caused the highest inhibition was not the same in all cases but was dependent on the *Fusarium* and host plant species.

INTRODUCTION

Polyphagous Shot Hole Borer (*Euwallacea* sp. I, "PSHB") and Kuroshio Shot Hole Borer (*Euwallacea* sp. II, "KSHB") are invasive ambrosia beetle species that are threatening a wide range of tree species found in various natural habitats, urban landscapes, and the commercial avocado groves in Southern California (Eskalen *et al.* 2013; O'Donnell *et al.* 2014, Kasson et al. 2013). Both beetle species are known to vector several obligately associated plant pathogenic fungal species as they bore networks of galleries in the xylem tissues of host plants (Freeman *et al.* 2012). As closely related species of ambrosia beetles to the tea shot hole borer (*Euwallacea fornicatus* Eichoff, "TSHB"), PSHB and KSHB are distinct from most ambrosia beetle species in that they colonize healthy, living hosts as opposed to the other ambrosia beetles' usual stressed or dead hosts (Batra 1967; Danthanarayana 1968; Hulcr et al. 2011). These invasive beetles

are thought to have originated from the south eastern region of Asia, and are known to be able to colonize and kill a wide range of woody plant species – 139 species of trees in California have been observed to be susceptible to colonization by these beetles, 41 of which are reproductive host species that allow the beetles to complete their life cycles within (Eskalen *et al.,* 2013, *http://eskalenlab.ucr.edu/avocado.html*). Among the reproductive host species are tree species commonly used in landscaping, such as the California sycamore (*Platanus racemosa*) and coast live oak (*Quercus agrifolia*), common species found in various natural riparian habitats such as castor plant (*Ricinus communis*), and economically important crop species such as avocado (*Persea americana*) (Eskalen *et al.,* 2013). The latter plant species' susceptibility to colonization by PSHB and KSHB is a critical concern to the California avocado industry. The wide host range of the beetles, combined with the ubiquitous distribution of host plant species in both urban and natural environments of Southern California have allowed the PSHB and KSHB to proliferate in their respective niches since the beetles' discovery in 2012. Whereas the beetle infestation was limited to the Los Angeles County in 2012, the beetles have since spread to the neighboring Ventura, Orange, Riverside, San Bernardino, and San Diego Counties as of 2016, highlighting the urgent need for a control strategy against the beetle-fungus complexes.

As ambrosia beetle species adapted to fungus-only diet, PSHB and KSHB share obligate mutualistic symbioses with their ambrosia fungi (Kasson *et al.* 2013). Like other ambrosia beetle species, PSHB and KSHB cultivate their ambrosia fungi within their galleries while actively maintaining the "fungal gardens" to prevent contamination and

overgrowth (Biedermann and Taborsky 2011). This behavior is similar to the fungus-farming found in the TSHB (Batra 1967; Beaver 1989), which is known to be obligately associated with the *Fusarium ambrosium* (Freeman *et al*. 2013). *F. ambrosium* conidia are deposited onto the gallery surface as the beetles bore during the gallery construction, and are actively cultivated by the beetles. This fungus serves as a source of nutrition for the beetles as it concentrates the nutrients from the plant tissues for the beetles to consume, while also infecting and spreading through the host vascular system (Beaver 1989). In PSHB and KSHB, this role is performed by *Fusarium euwallaceae* and *Fusarium kuroshium* respectively (Freeman *et al.* 2013; F. Na 2016, unpublished data). These related but genetically distinct fusaria cause dieback symptoms in the host plants when the combination of physical damage to the xylem vessels through gallery boring and severe infection in the xylem tissues render the xylem vessels unable to adequately transport nutrient and water (Eskalen *et al.* 2013). This dieback gradually worsens as more beetles reproduce and the newly emerged beetles further colonize xylem tissues in host. Continued proliferation of beetles in a plant leads to host mortality when the infected vascular tissues in the infested plant are no longer able to adequately transport water and nutrients to sustain life (Freeman *et al.* 2013).

While the damage caused to the host vascular system by the boring of galleries in xylem alone can be significant and contributes to the dieback symptoms in host, the dieback symptoms likely occur due to the combination of the damage caused by the beetles' boring and by the infection of xylem tissues by the beetle-symbiotic fungi. Three species of such fungi are known to exist in association with PSHB, *Fusarium*

euwallaceae, Graphium euwallaceae, and Paracremonium pembeum (Lynch *et al.* 2016), and two species are known to be associated with KSHB, *Fusarium kuroshium* and *Graphium kuroshium* (F. Na 2016, manuscript in preparation). These fungal species have been isolated from both the mycangia of PSHB and the surfaces within beetle galleries, and each fungus has been found to independently cause disease symptoms in box elder (*Acer negundo*) and avocado (*Persea americana*) in previous studies (Lynch *et al.* 2016; F. Na 2016 unpublished data). Though the exact roles of the beetle-associated fungal species are not yet well understood, Freeman *et al.* (2015) has observed that *G. euwallaceae* was predominantly present in the guts of the PSHB larvae, while *F. euwallaceae* was predominantly present in the adult females. The beetles have also been observed to be capable of completing their life cycles while fed *F. euwallaceae* or *G. euwallaceae* exclusively, while unable to survive on *P. pembeum* alone (Freeman *et al.* 2015). This observation has led to the current hypotheses for the roles of the 3 fungal species associated with the PSHB: *F. euwallaceae* serves as the primary food source of adult beetles, and is required for the establishment of new galleries; *G. euwallaceae* serves as the primary food source for the juveniles in the gallery until adulthood; *P. pembeum* has an unknown secondary function that depends on the host plant species being colonized (Freeman *et al.* 2015; Lynch *et al.* 2016). These hypotheses are thought to apply to the fungal associates of the closely related KSHB as well, with the *F. kuroshium* serving as the primary source of food for adult beetles and *G. euwallaceae* serving as the primary source of food for the juveniles. As such, the assumed role of *F. euwallaceae* and *F. kuroshium* as requirements for the establishment of new galleries

make the fungal associates perfect targets of preventive treatments in areas with high risk of future PSHB/KSHB infestation.

We hypothesized that methods to prevent successful *Fusarium* colonizations in the xylem vessels in host plants can be utilized as preventive treatments for the control of PSHB and KSHB in areas with high risk of future beetle infestation. Preventing the ambrosial *Fusarium* from successfully colonizing the host plants should prevent further spread of the beetles to new hosts, as the lack of ambrosial *Fusarium* species should limit the success of the newly emerged beetles in establishing new galleries. Use of this method in areas with high risk of infestation could create "buffer zones" that the beetles cannot cross, effectively quarantining the beetles from entering the neighboring uninfested areas.

In order to preventively control *Fusarium euwallaceae* and *Fusarium kuroshium* within host, a treatment method that can prevent successful colonization stably over time may be appropriate to apply. Sustained inhibition of the primary nutrient source of the ambrosia beetles should not only prevent the development of Fusarium Dieback symptoms in host plants, but also prevent successful colonization. We hypothesized that this may be accomplished by utilizing naturally occurring plant-endophytic microorganisms in xylems of host plant species as injectable biological control agents. The use of plant-endophytic bacteria in the biological control of pathogenic fungal species have been shown to be effective in previous studies involving various host-pathogen species, such as tropical angiosperm and *Phytophthora* sp. (Arnold *et al.* 2003); live oak and *C. fagacearum* (Brooks *et al.* 1994); cucumber and *F. oxysporum* (Cao *et al.*

2011); cotton and *F. oxysporum* (Chen *et al.* 1995); tomato and *F. oxysporum* (Nejad and Johnson 2000). Several of these studies suggest that this strategy can be utilized to inhibit pathogenic fungal species belonging to the *Fusarium* genus (Cao *et al.* 2011; Chen *et al.* 1995; Nejad and Johnson 2000). Plant-endophytic bacteria as biological control that directly targets the xylem-infecting *F. euwallaceae* and *F. kuroshium* may be advantageous for use in creating protective layers for the wood tissues of uninfested plants in areas with high risk of PSHB/KSHB infestation, as the endophytic nature of the organisms should allow them to persist in plant tissues, providing lasting protection against the pathogenic fungi.

In this study, we obtained endophytic bacteria from the wood tissues collected from disease-escaped individuals belonging to susceptible plant host species, then evaluated their efficacies in inhibiting *Fusarium euwallaceae* and *Fusarium kuroshium in vitro* and *in planta.* We show that the endophytic *Bacillus subtilis* are capable of inhibiting both fusaria *in vitro,* and that they were able to reduce the severity of symptoms in *Fusarium*-infected plants when applied as injection treatments. The capability of the bacterial treatments to remain viable within plant tissues over a period of 30 days show potential for the bacterial endophytes as lasting preventive treatment for the PSHB-associated *Fusarium euwallaceae* and the KSHB-associated *Fusarium kuroshium.*

MATERIALS AND METHODS

Endophytic bacteria collection - Endophytic bacterial isolates used in this study were obtained from the wood tissue samples of California sycamore (*Platanus racemosa*) and

Avocado (*Persea americana*, cv. Hass) within several PSHB or KSHB-infested locations in Los Angeles and San Diego Counties. Total of 20 of each tree species that were harboring relatively lower number of beetle galleries compared to other nearby trees in the sampling areas were visually identified and selected for sampling. A hand drill was used to obtain small wood samples 2 cm in diameter and 2.5 cm in depth from the trunks of California sycamore, and a pruning shear was used to cut segments from branches of avocado. One xylem sample was taken from each tree, and each sample was transported to laboratory in a separate polyethylene bag. These samples were flame-sterilized then cut into small wood chips, and the chips from each sample was macerated in 20ml of sterile water using a mortar and pestle. The resulting suspensions were transferred to 50 ml centrifuge tubes and incubated in 25°C overnight, then 100 µl of each suspension was pipetted and spread with sterile glass rods in petri plates containing nutrient agar for culturing bacteria, or potato dextrose agar amended with tetracycline for culturing fungi. Cultures were incubated in 25°C for 5 days, and individual bacterial colonies from these plates were transferred to fresh nutrient agar plates then stored in 1.5ml centrifuge tubes containing sterile 30% glycerol or water for future use.

Bacterial isolates from xylem samples were screened *in vitro* for the ability to inhibit *Fusarium euwallaceae* using dual culture methods described by Leelasuphakul *et al.* (2008). Bacterial isolates were determined to be inhibiting *Fusarium euwallaceae* if they formed zones of inhibition in the space between the bacterial colony and the fungal colony in dual culture plates after incubation. All bacterial isolates were transferred to plates containing nutrient agar, in which the bacterial inoculation points were arranged so

that 5 colonies could be screened simultaneously with 1 *F. euwallaceae* colony in each plate. Bacteria were first transferred to 5 points along the outer edges of each plate using sterile tooth picks, then incubated in 25°C overnight. A *F. euwallaceae* agar plug was added to the center of each plate the next day, then the cultures were further incubated in 25°C for 5 days. Every bacterial colony that developed a zone of inhibition was selected for further evaluation to determine the isolate's efficacy in inhibition of *F. euwallaceae.* The inhibiting bacteria were subsequently identified by DNA sequences of the bacterial 16S ribosomal RNA.

Efficacy of inhibition - The efficacy of inhibition by each of the bacterial isolates was evaluated by measuring *F. euwallaceae* and *F. kuroshium*. colony radii after incubation in dual culture with the bacteria. The bacterial efficacy of inhibition was measured as percent inhibition of the fungal colonies, and the percent inhibition was calculated by comparing the colony radii in fungal-bacterial dual cultures to those of the *Fusarium* colonies in control plates without bacteria (Chaurasia *et al.* 1997).

A slightly modified dual culture protocol compared to that used during the bacterial screening process (Leelasuphakul *et al.* 2008) was used, in which the all of the 5 bacterial inoculation points in each plate were arranged to be 3.5 cm away from the center of the plate. The center agar plugs containing *F. euwallaceae* or *Fusarium kuroshium*. were cut from 7-day old cultures of 4 different isolates of the *Fusarium* spp., 2 isolates from each *Fusarium* species. A sterile cork borer 5mm in diameter was used to create agar plugs with fungal mycelium, and these plugs were placed on the center of plates with mycelium-side down one day after bacterial inoculation. In control plates, the

5 bacterial inoculation points along the periphery of the plates were pierced with sterile toothpicks. 4 replicates per bacterial isolate per fungal isolate was used in each trial, and two trials were conducted. Cultures were incubated in 25°C for 7 days, and the radius of each fungal colony as it grew towards each bacterial colony were measured. No fungal colony physically reached the bacterial inoculation points within 7 days, and the radii of the fungal colonies that were inhibited by the bacteria were compared to the mean colony radius of *Fusarium euwallaceae* or *Fusarium* sp. in control plates, and percent inhibition was calculated from each radius by using the following formula: (Control Radius – Inhibited Radius) / Control Radius * 100 = Percent Inhibition.

Greenhouse bacterial injection - A preliminary experiment was first conducted to investigate the efficacy of *B. subtilis* as preventive or curative foliar spray treatment of *F. euwallaceae.* A total of 60 avocado branches were sprayed with *B. subtilis* in water, *B. subtilis* in surfactant-penetrant, surfactant only, or water only. Branches were either inoculated with *F. euwallaceae* 3 days after treatment with bacteria or 3 days prior to treatment with bacteria, in order to compare the symptom severity between the preventively treated trees and the curatively treated trees. All branches were incubated for 14 days, then the lengths of lesions originating from *F. euwallaceae* inoculation point were measured.

A greenhouse study was conducted using methods described by Eastwell *et al.* (2006) with the bacterial isolates that showed the highest inhibition of *F. euwallaceae* and *F. kuroshium* during the percent inhibition experiment. The bacterial isolates selected consisted of 2 highest-performing isolates of *Bacillus* sp. obtained from California

sycamore (E6, E9) and 2 highest-performing isolates from avocado (E20, E21). The bacteria were cultured on nutrient agar in 25°C for 2 days, and one bacterial colony for each isolate was transferred to a 15 ml test tube containing 1 ml of nutrient broth amended with 2% NaCl. The nutrient broths were incubated overnight in an incubator shaker set to 30 ^{o}C, 130 RPM. To increase the total volume of each bacterial suspension, 500 µl from each of the broths was then transferred to a larger flask containing 20 ml of nutrient broth amended with 2% NaCl, and the flasks were incubated again overnight in the incubator shaker. 1 ml from each nutrient broth was transferred to 1.5 ml centrifuge tubes prior to centrifuging at 4,000 RCF to pellet the bacteria. Supernatants containing nutrient broth was removed from the centrifuge tubes, and 1ml of sterile water was added to each. All tubes were centrifuged once more, then supernatants were removed again in order to completely remove nutrient broth from the tubes. The pelleted bacteria in each tube were resuspended in 1ml of sterile 0.085% NaCl in water then mixed in a vortex mixer (Eastwell *et al.* 2006). The finished bacterial suspensions were checked for viability by spread plating on nutrient agar, and were diluted to 5*10^7 CFU/ml.

The bacterial suspensions were injected as preventive treatments in 100 2-year old avocado seedlings and 100 3-year old California sycamore seedlings over 2 experimental trials. During each trial, each bacterial treatment was applied to 10 avocado stems and 10 California sycamore stems, and 5 among each host species were infected with *F. euwallaceae*, while the remaining 5 were infected with *Fusarium* sp. The seedlings were bacterized with the prepared 5*10^7 CFU/ml bacterial suspensions containing the endophytic bacteria with the highest *in vitro* inhibition of *F. euwallaceae* and *F.*

kuroshium, using methods described by Eastwell *et al.* (2006). 10 µl droplets of the bacterial suspensions were pipetted onto the epidermal tissues of California sycamore and avocado stems in each inoculation point, each stem containing 4 equidistant bacterial inoculation points and the center pathogen inoculation point along a 10 cm region of each stem (Fig 2.2). Each droplet was pierced using a sterile 22-gauge needle to allow the bacterial suspension to be absorbed into the xylem tissues. Control plants were injected with sterile 0.085% NaCl in the bacterial inoculation points. Capillary action within the xylem vessels generally allowed the suspensions to be absorbed immediately upon piercing with the needles in most plants, while others absorbed over several minutes or required additional needle pierces. Each point of inoculation was wrapped with sterile Parafilm M (Bemis NA) once all liquid was absorbed through the wound.

The plants were infected with *F.euwallaceae* or *F. kuroshium* 7 days after bacterization. Conidial suspensions containing *F. euwallaceae* or *F. kuroshium* conidia were used to infect the bacterized plants. 1 isolate from each *Fusarium* isolate was used. *F. euwallaceae* and *F. kuroshium* were cultured on potato dextrose agar and were incubated in 25°C for 14 days in constant darkness. The cultures were then flooded with 3ml of sterile water, and a sterile glass rod was used to agitate the mycelia in order to release the conidia into the water. The resulting liquids were pipetted from the plate into sterile filter papers, allowing only the *Fusarium* conidia and water to fall into 1.5ml centrifuge tubes. The conidial concentrations in the filtrates were measured using a hemocytometer, and the filtrates were diluted to the final conidial concentration of 5*10^6 CFU/ml. 10 µl of *F. euwallaceae* or *F. kuroshium* conidial suspension was

pipetted onto each pathogen inoculation point in the stems of bacterized avocado and California sycamore. Droplets were pierced with a sterile 22-gauge needle, and the wounds were similarly wrapped with sterile Parafilm M once the liquids were completely absorbed.

The treated plant stems were collected from greenhouse 4 weeks after the fungal inoculation. A 30 cm segment from each treated stem was cut from the plants and brought to lab for analysis. The stem segments were surface sterilized by exposing briefly over open flame, and the plant tissues above the xylem layer were peeled back with a sterile knife to reveal the xylem tissues. The lengths of xylem discoloration originating from the pathogen inoculation points were measured, and small samples of xylem were taken from pathogen inoculation point, two outer bacteria inoculation points, two lesion tips, and two points 1cm away from the lesion tips. These samples were plated in duplicates on tetracycline-amended potato dextrose agar and plain nutrient agar. After 7 days of incubation at 25°C, the presence of *Fusarium euwallaceae* or *Fusarium* sp. in each xylem sample was noted, as well as the presence of *Bacillus* sp. in each xylem sample in nutrient agar.

Statistical analyses – Statistical analyses were performed using JMP 11.0.0 (SAS Institute Inc., Cary, North Carolina). Regression modeling was used to compare the percent inhibition data obtained from *F. euwallaceae* and *F. kuroshium* colonies inhibited by *B. subtilis*, and significant interaction was detected between bacterial isolates used ($p<0.0001$). Differences in percent inhibition data among *Fusarium* and *Bacillus* isolates were compared using Tukey's honest significant difference (HSD) test at $\alpha = 0.05$.

Regression modeling was used to compare the lesion sizes in bacterized California sycamore and avocado, and significant interaction was detected between bacterial isolate used ($p<0.0001$), but no significant interaction was detected in *Fusarium* isolates used ($p=0.1257$) and host tree species used ($p=0.0749$). Lesion length data was transformed using 1/ sqrt(x) transformation, and normality was checked using Shapiro-Wilk test. The transformed data was compared by Tukey's honest significant difference test at $\alpha = 0.05$.

RESULTS

Endophytic bacteria collection - A total of 182 bacterial isolates were obtained from avocado and California sycamore wood samples. Of the 182 isolates, 15 were found to visibly inhibit *Fusarium euwallaceae in vitro.* 13 among the 15 potential biological control agents originated from California sycamore wood samples taken from El Cajon, CA, and the remaining two isolates originated from avocado wood samples taken from San Marino, CA. Bacterial 16S rRNA sequences identified the 15 bacterial isolates to be *Bacillus* spp.

Efficacy of inhibition - The colony radii of 7-day old *Fusarium euwallaceae* and *Fusarium kuroshium* that were simultaneously cultured with the bacterial endophytes in the dual culture plates were found to be significantly reduced compared to the uninhibited control in all colonies challenged with the bacteria (Fig 2.1.2). Reduction in *F. euwallaceae* and *F. kuroshium* colony radii compared to control after 7 days of incubation ranged from 41.9% to 51.5% across all bacterial isolates used. Mean radii of

uninhibited *F. euwallaceae* and *F. kuroshium* colonies grown on nutrient agar for 7 days in 25°C were 3.04 cm and 3.13 cm respectively. No significant differences in colony size was detected between all *F. euwallaceae* and *F. kuroshium* isolates in control plates. Percent inhibition significantly differed between bacterial isolates, with 5 of the 15 isolates consistently showing significantly higher percent inhibition when challenged with either of the *Fusarium* species (Fig 2.1.1). *In vitro* inhibition efficacy of each bacterial isolate was generally conserved across both *Fusarium* species; the 5 bacterial isolates with the highest mean percent inhibition of *F. euwallaceae* were also found to be the 5 highest in mean percent inhibition of *F. kuroshium*. No statistically significant differences between the colony radii of *Bacillus*-inhibited *F. euwallaceae* and *F. kuroshium* were detected. The California sycamore-endophytic E6 and E9 showed the greatest percent inhibition among all other California sycamore endophytes, and the avocado-endophytic E20 and E21 showed percent inhibition comparable to those of E6 and E9.

Greenhouse Bacterial Injection - The preliminary experiment showed that the average *F. euwallaceae* lesion lengths in branches sprayed with *B. subtilis* suspensions in either water or surfactant-penetrant were lower than those in branches treated with water or surfactant-penetrant only, as long as the treatments were applied in prior to pathogen inoculation. Presence of *Fusarium*-inhibiting bacterial colonies were confirmed in the nutrient agar plates containing wood samples taken from the treated branches.

Bacterial injections in avocado and California sycamore showed significant effect in reducing severity of the xylem staining caused by *Fusarium euwallaceae* and

Fusarium kuroshium infection in three of the four host-pathogen combinations used. None of the bacterial treatments was found to significantly reduce lesion lengths in avocado infected with *F. kuroshium.* Each bacterial isolate used during injection was found to significantly reduce lesion lengths in at least one plant host – pathogen combination at $\alpha = 0.05$, though no single bacterial isolate was found to significantly reduce lesion lengths in all host – pathogen combinations. (Fig. 2.3). The bacterial isolate E6 was found to significantly reduce fungal lesion sizes only in avocado challenged with *F. euwallaceae* ($p = 0.0143$); its effects in all other host-pathogen combinations were not statistically significant. Isolate E9 was found to significantly reduce lesion sizes in avocado challenged with *F. euwallaceae* ($p = 0.0058$), California sycamore challenged with *F. euwallaceae* ($p = 0.0003$), and California sycamore challenged with *F. kuroshium* ($p = 0.0011$). Isolates E20 and E21 were effective in significantly reducing lesion sizes in avocado infected with *F. euwallaceae* ($p = 0.0167$ and 0.0493, respectively) and California sycamore infected with *F. kuroshium* ($p < 0.0001$ for both).

Both *Fusarium* spp. and *Bacillus* sp. were recovered from xylem samples taken from bacterial injection-treated plants, while only *Fusarium* spp. were recovered from xylem samples collected from water-injected plants. No *Bacillus* colonies were found on plates containing xylem samples collected from water-injected control plants, and no bacterial colonies were found on PDA-tetracycline plates. Recovery data showed that *Fusarium* recovery rate from samples decreased as the sampling distance from fungal inoculation point increased (Fig. 2.4.3). Bacterial recovery showed similar trends – recovery rate generally decreased as the sampling distance from the bacterial injection

points increased. Coexistence of *Fusarium* and *Bacillus* colonies originating from the same xylem samples in nutrient agar was noted in few samples. Such *Fusarium* spp. colonies were highly inhibited by the neighboring *Bacillus* colonies, but were able to germinate and form aerial mycelium.

DISCUSSION

The results from this study showed that some of the endophytic bacterial isolates obtained from the xylem tissues of PSHB-susceptible host tree species were effective in reducing the severity of the symptoms caused by the PSHB and KSHB-associated *Fusarium euwallaceae* and *Fusarium kuroshium in planta*, though the effectiveness of each bacterial isolate differed depending on the host-pathogen combination it was used in. At least one bacterial isolate was found to be effective in reducing the xylem lesions caused by *F. kuroshium* in both host plant species and by *F. euwallaceae* in avocado, while no bacterial isolate was found to be effective in reducing the sizes of lesions caused by *F. kuroshium* in avocado. Though the recovery frequency data obtained from the bacterized avocado and California sycamore did not show noticeable reduction in *Fusarium* recovery frequency, the significant reductions in fungal lesion length observed in most bacterized plants show promise for the plant-endophytic *Bacillus* spp. as biological control of *Fusarium euwallaceae* and *Fusarium kuroshium.*

All 15 bacterial isolates that showed visible inhibition of *Fusarium euwallaceae in vitro* during the initial dual culture screen for *Fusarium*-inhibiting endophytic bacteria were found to be species belonging to the *Bacillus subtilis* species complex (Rooney *et*

al. 2009). The portion of 16S rRNA gene used to identify these bacteria was not able to resolve the identity of each bacterial isolate to a single species, thus all 15 bacterial isolates were able to be putatively identified as *Bacillus subtilis* by BLASTN search. Though the endophytic bacterial communities within the xylem of avocado and California sycamore have not yet been studied extensively, *Bacillus* species are known to occur commonly in association with plants; species belonging to the genus have been observed to occur in various plant parts across many plant species, including the rhizosphere of avocado (Cazorla *et al.* 2007; Lodewyckx *et al.* 2002; Misaghi and Donndelinger 1990; Sturz *et al.* 1996). As such, the presence of *Bacillus* sp. in avocado and California sycamore was not unexpected. The lack of any other endophytic bacterial species that could inhibit *Fusarium euwallaceae in vitro* was surprising, however. Species belonging to the genus *Pseudomonas,* along with those belonging to the genus *Bacillus,* were expected to be present in the samples. Pseudomonads are commonly found as endophytic bacterial associates of plants, as *Pseudomonas* species are known to be commonly present as endophytic bacterial associates of plants (Lodewyckx *et al.* 2002). Some *Pseudomonas* species have been shown to be effective as biological control of fungal diseases, such as the plant-endophytic species *Pseudomonas fluorescens. P. fluorescens* is known to produce antibiotic compounds (Haas and Keel 2003), and has been observed to be effective in the biological control of *Fusarium oxysporum,* the causal agent of the Fusarium wilt (Raaijmakers *et al.* 1995; Lemanceau and Alabouvette 1991). As pseudomonads are known to grow in the growth medium used during the screen, the absence of these bacteria was likely due to the limited sampling from few locations.

Investigation of *P. fluorescens* as potential biological control of *F. euwallaceae* and *F. kuroshium,* and comparison between *P. fluorescens* and *Bacillus* spp. as biological control of the beetle-associated fusaria may be an interesting direction for future research.

Percent inhibition data, calculated as efficacies in the inhibition of *F. euwallaceae* and *F. kuroshium* by the 15 plant-endophytic bacterial isolates in dual cultures, showed that all of the bacterial isolates were able to reduce the colony radii of *F. euwallaceae* and *F. kuroshium* by more than 40%, and that some of the bacterial isolates among the 15 isolates used had significantly higher inhibition efficacy compared to others. The appearance of defined zones of inhibition between the bacterial colonies and the *Fusarium* colonies suggests the presence of diffusible compounds produced by the endophytic *Bacillus subtilis* that are capable of inhibiting *F. euwallaceae* and *F. kuroshium*. The antifungal properties of *Bacillus subtilis* has been studied extensively (Nagórska *et al.* 2007; Ongena and Jacques 2008, Stein 2015), and *B. subtilis* has been found to be effective as biological control of *Fusarium* fungal diseases of plants, such as the Fusarium wilt (Baysal *et al.* 2008; Cao *et al.* 2011; El-Hassan and Gowen 2006). The diffusible compounds that inhibited *F. euwallaceae* and *F. kuroshium* are likely antifungal lipopeptides belonging to the iturin, fengycin, and surfactin families, or any combinations of these compounds. These compounds have been observed to possess strong antifungal properties due to their abilities to permeabilize the cell membrane structures (Nagórska *et al.* 2007; Ongene and Jacques 2008) and have been hypothesized to be the diffusible antifungal metabolites responsible for *in vitro* fungal inhibition (Grover *et al.* 2009). The production of the different lipopeptides can differ even among

isolates of bacteria within *B. subtilis* species (Carzola *et al.* 2007), and this difference in lipopeptide production may have been a contributing factor to the difference in inhibition efficacy observed between the isolates used during the percent inhibition study.

While the antifungal lipopeptides are known to contribute to inhibition of fungal species, it is not the only known factor to the overall inhibition. For future studies, it may be necessary to take the effects of volatile compounds produced by *B. subtilis* and other closely related species into consideration during the *in vitro* evaluation. Volatile compounds produced by *Bacillus subtilis* and other closely related species have been found to have profound effects in fungal growth of various fungal species in dual-culture (Chaurasia *et al.* 2005; Fiddaman and Rossall 1993; Yuan *et al.* 2012), and the effects of volatiles produced by the colonies may have affected the sizes of the *Fusarium* colonies for during the efficacy of inhibition assay. It is currently not well understood if the volatile compounds produced by the endophytic bacteria in xylem have effects on fungal colonization in plants. Most studies done regarding this topic are related to *B. subtilis* as a species associated with the rhizospheres of plants, though some suggest that the bacteria may have effects on disease development in xylem by pathogenic fungi: Fiddaman and Rossall (1994) found that *B. subtilis* in the surface of roots produce small amounts of volatile compounds that inhibit *Rhizoctonia solani*, and Ryu *et al.* (2004) found that *B. subtilis* and *B. amyloliquefaciens* in plant roots produce volatile compounds that trigger induced systemic resistance (ISR) in plants. ISR likely has a large impact on fungal colonization in plants treated with biocontrol bacteria (Kloepper *et al.* 2004), and a future studies to further elucidate the relationship between direct effects of the endophytic

bacteria on fungal colonization and the effects on the fungi by bacteria-induced systemic resistance may be needed.

The greenhouse bacterial injection experiment showed that the bacterial injections have significant effects in reducing the sizes of fungal lesions in *Persea americana* and *Platanus racemosa*, except in avocado infected with *Fusarium kuroshium.* Interestingly, the results obtained from avocado and California sycamore during the *in planta* bacterial injection study were not consistent across the two host plant species. The results from this experiment suggest that different species of host plants benefit differently from the endophytic bacteria injections, and that the severity of symptoms caused by *F. euwallaceae* and *F. kuroshium* differ between the two hosts. The differences in the effectiveness of treatments in both host species may be due to the existence of factors that were not accounted for during the experiment, such as the abilities of the bacterial treatments to elicit ISR in xylem (Kloepper *et al.* 2004), and the volatile compounds emitted by the bacteria (Chaurasia *et al.* 2005).

The recovery of bacteria only from bacteria-treated plants showed that the injected bacteria were able to persist in the plants, and that the bacteria were able to move within the plants. The persistence and translocation observed in the injected bacteria show great promise for the bacteria as effective biological control agents for *F. euwallaceae* and *F. kuroshium.* While we were unable to verify whether the bacterial load within the plants grew or fell over time, previous studies that have shown that bacteria are capable of reproducing (Chen *et al.* 1995) and moving in the xylem tissues (Brooks *et al.* 1994) after artificial inoculation suggest that the endophytic *B. subtilis*

likely also reproduced in the plants. The recovery data also showed that *Fusarium* recovery frequency in a given sampling location is generally not affected by the presence of *B. subtilis* in the same location, whether or not the bacteria had significant effects on the sizes of the fungal lesions. This result suggests that the bacteria are producing compounds that are not fungicidal but fungistatic. We anecdotally noted that in few plates containing xylem samples from bacteria-treated stems, both *B. subtilis* colony and *Fusarium* colonies were growing out simultaneously from a single xylem sample. The fungal colony was severely inhibited and consisted mostly of aerial mycelium, but it nevertheless grew from a xylem sample that also contained the injected bacteria. If the bacteria were producing fungicidal compounds, then the recovery frequency data would show that the *Fusarium* recovery is lower where the bacteria can be isolated, which was not the case. Future studies that address the current unknown aspects of *F. euwallaceae* and *F. kuroshium* pathogenicity in various hosts, as well as those that elucidate the nature of inhibition by the endophytic bacterial isolates obtained in this study will be greatly beneficial to the efforts to develop effective treatment methods to control the fungal associates of the invasive ambrosia beetles.

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Table 2.1 Bacterial isolates used in this study

Isolate ID	Location	Host	Identity
E1	El Cajon, CA	*Platanus racemosa*	*B. subtilis*
E2	El Cajon, CA	*Platanus racemosa*	*B. subtilis*
E3	El Cajon, CA	*Platanus racemosa*	*B. subtilis*
E4	El Cajon, CA	*Platanus racemosa*	*B. subtilis*
E5	El Cajon, CA	*Platanus racemosa*	*B. subtilis*
E6	El Cajon, CA	*Platanus racemosa*	*B. subtilis*
E7	El Cajon, CA	*Platanus racemosa*	*B. subtilis*
E8	El Cajon, CA	*Platanus racemosa*	*B. subtilis*
E9	El Cajon, CA	*Platanus racemosa*	*B. subtilis*
E10	El Cajon, CA	*Platanus racemosa*	*B. subtilis*
E11	El Cajon, CA	*Platanus racemosa*	*B. subtilis*
E12	El Cajon, CA	*Platanus racemosa*	*B. subtilis*
E13	El Cajon, CA	*Platanus racemosa*	*B. subtilis*
E20	San Marino, CA	*Persea americana*	*B. subtilis*
E21	San Marino, CA	*Persea americana*	*B. subtilis*

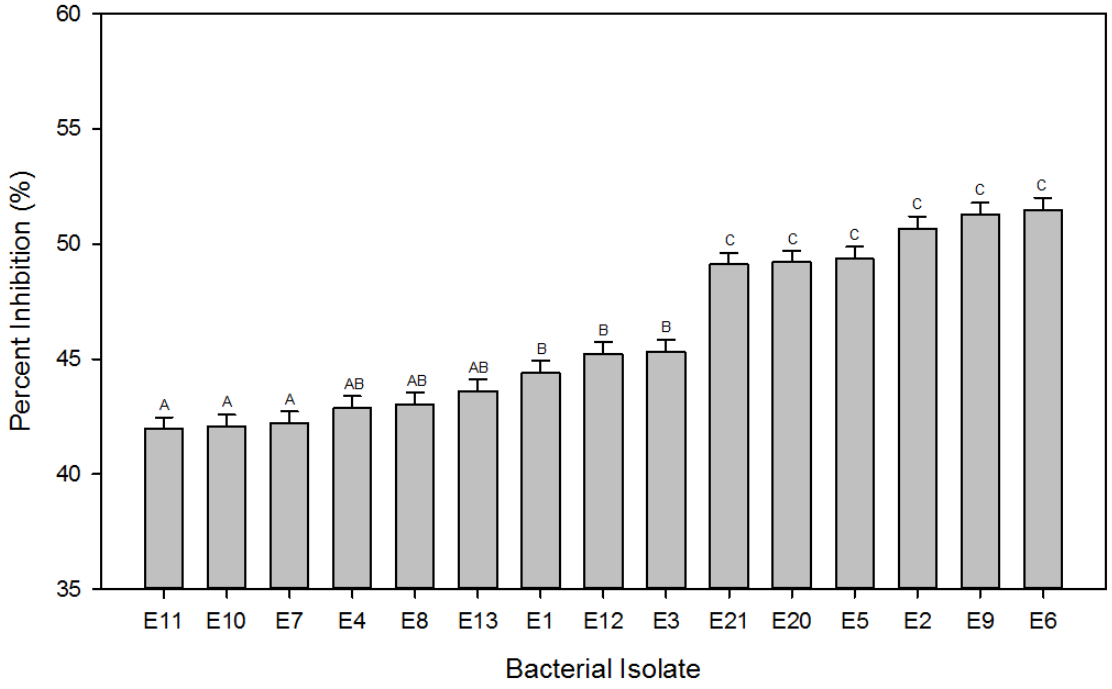

Figure 2.1.1. Inhibition efficacy of endophytic bacterial isolates, calculated as percent inhibition in the mean *F. euwallaceae* and *F. kuroshium* colony radii compared to uninhibited control in dual culture plates containing the endophytic bacterial isolates. Cultures were incubated in 25°C for 7 days prior to measuring. Percent inhibition was calculated using the formula: (A – B) / A *100, where A = colony radius in control, B = inhibited colony radius. Vertical lines represent standard error of mean. Statistical analysis was performed using Tukey's honest significant difference (HSD) test at α = 0.05. Levels connected by the same letter are not significantly different.

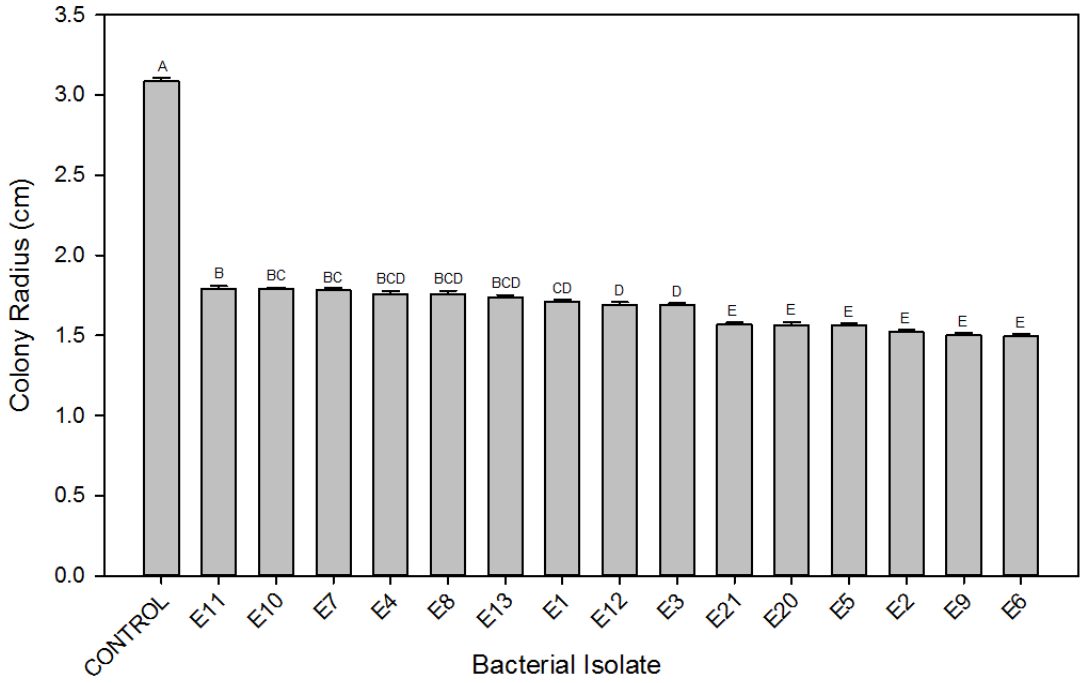

Figure 2.1.2. Mean *F. euwallaceae* colony radii in dual culture plates containing colonies of endophytic bacterial isolates. Cultures were incubated in 25°C for 7 days prior to measuring. Vertical lines represent standard error of mean. Statistical analysis was performed using Tukey's honest significant difference (HSD) test at $\alpha = 0.05$. Levels connected by the same letter are not significantly different.

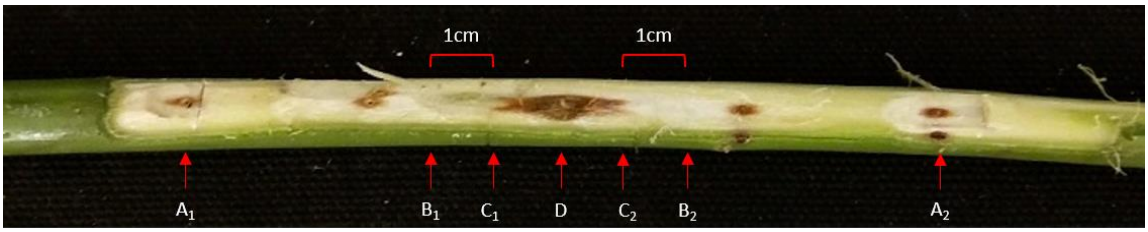

Figure 2.2. Diagram showing the layout of various injection points and sampling locations in a bacterized avocado stem infected with *F. euwallaceae.* Treatments were applied to four points along 10 cm segments within stems, two points on either side of the center pathogen inoculation point (D). Each point was distanced 2 cm away from each other. Samples were taken from (A_1), (A_2), (B_1), (B_2), (C_1), and (C_2) for bacterial or fungal recovery. ($A_{1,2}$): Treatment injection, ($B_{1,2}$): Lesion tip + 1cm, ($C_{1,2}$): Lesion tip, (D): Fungal injection.

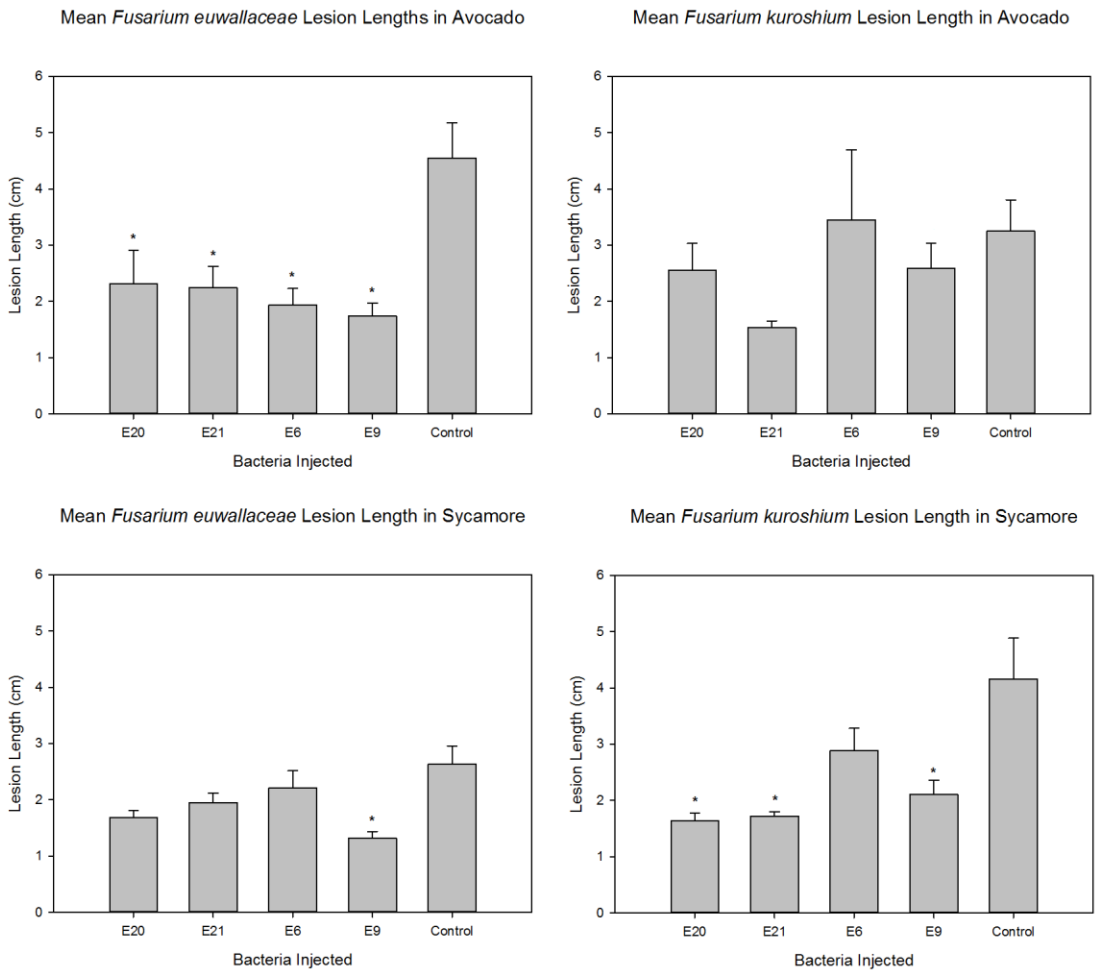

Figure 2.3. Mean lengths of *F. euwallaceae* and *F. kuroshium* lesions in xylem tissues of avocado (*P. americana*) and sycamore (*P. racemosa*) treated with endophytic bacterial injections or sterile water. Lesion lengths were measured 30 days after pathogen inoculation. Vertical lines represent standard error of mean. Statistical analysis was performed using Tukey's honest significant difference (HSD) test at $\alpha = 0.05$. Asterisks denote significant reduction of lesion length of control.

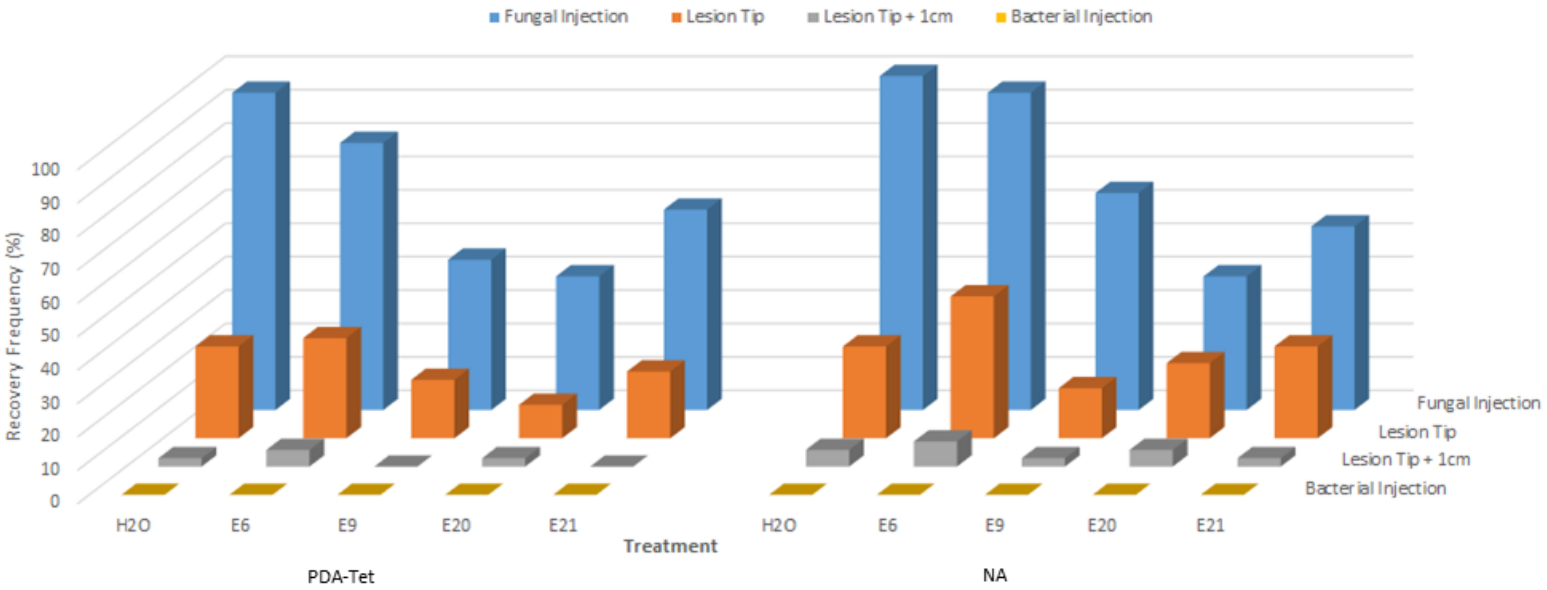

Figure 2.4.1. Recovery frequency of *F. euwallaceae* and *F. kuroshium* from tetracycline-amended potato dextrose agar and nutrient agar containing xylem samples taken from California sycamore stems. Xylem samples were taken from various points within a 10 cm segment in stems.

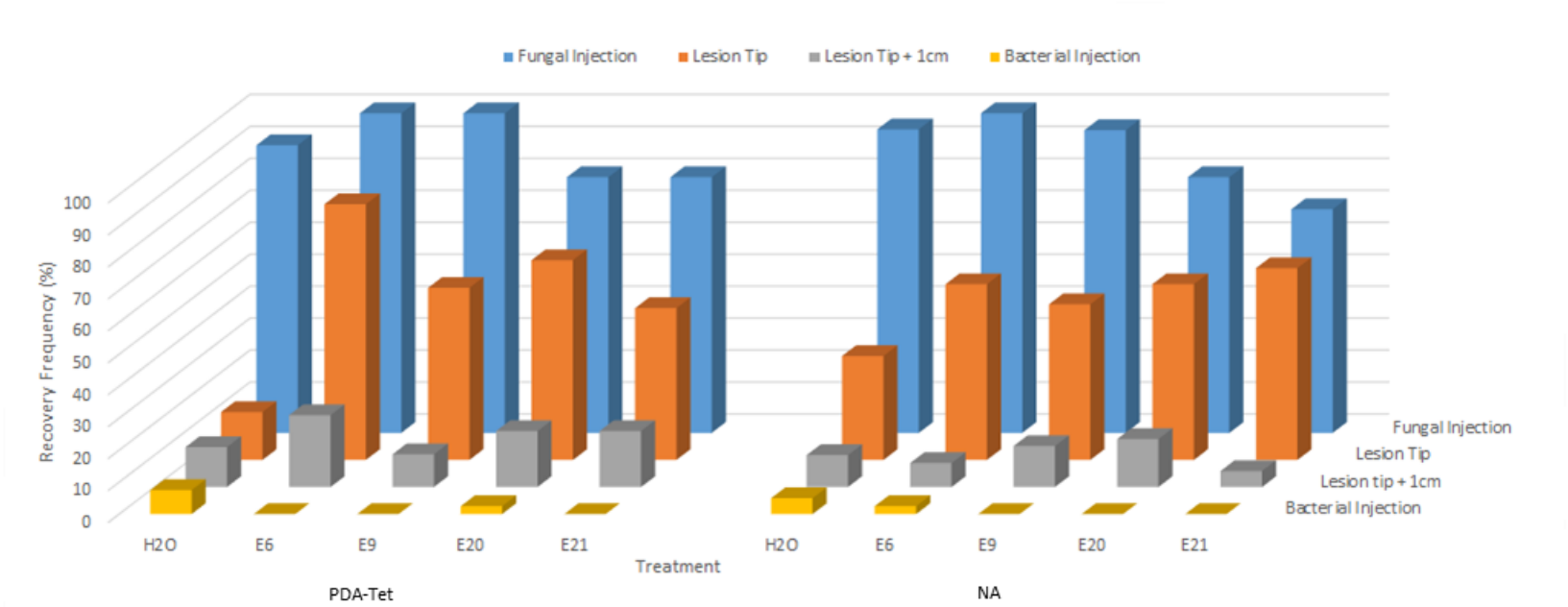

Figure 2.4.2. Recovery frequency of *F. euwallaceae* and *F. kuroshium* from tetracycline-amended potato dextrose agar and nutrient agar containing xylem samples taken from avocado stems. Xylem samples were taken from various points within a 10 cm segment in stems.

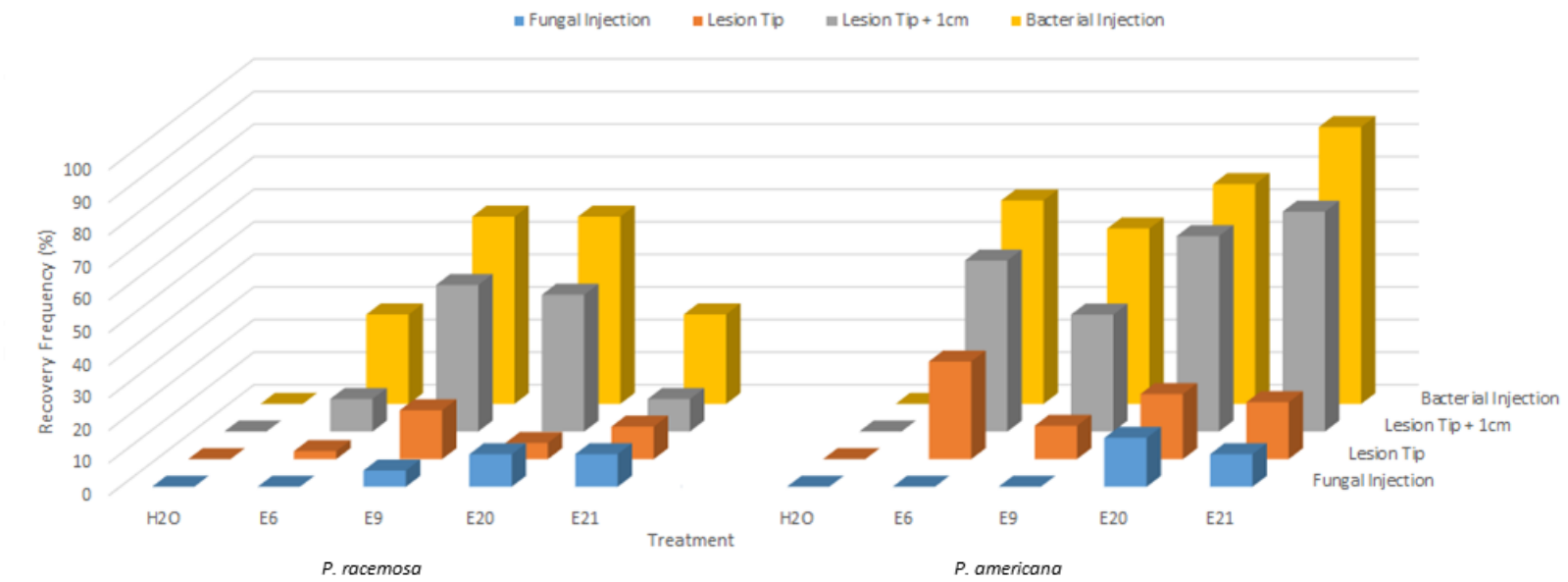

Figure 2.4.3. Recovery frequency of *Bacillus* sp. from avocado and California sycamore stems treated with bacterial injection or sterile water. Xylem samples were taken from various points within a 10 cm segment in stems.