UNIVERSITY OF CALIFORNIA
RIVERSIDE

Diagnostic Detection, Biology, and Management of Invasive Shot Hole Borers and Fusarium Dieback in California

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Plant Pathology

by

Joseph D Carrillo

March 2019

Dissertation Committee:
Dr. Akif Eskalen, Co-Chairperson
Dr. Jason E. Stajich, Co-Chairperson
Dr. Richard Stouthamer
Dr. Georgios Vidalakis

The Dissertation of Joseph D Carrillo is approved:

_____

_____

_____

Committee Co-Chairperson

_____

Committee Co-Chairperson

University of California, Riverside

ACKNOWLEDGEMENTS

Financial support for this work was provided by the grants from California Avocado Commission (CAC66013-85), CDFA Specialty Crop Block Grant Program (SCB16051), California Association of Nurseries and Garden Centers as well as a fellowship from the Los Angeles Center for Urban Natural Resources and Sustainability. Chapter III was done in collaboration with Christine Dodge from the Entomology Department and is a therefore a shared chapter.

I would like to first thank my advisor, Dr. Akif Eskalen, for accepting me into the lab and mentoring me, exposing me to robust field work, and allowing me to be creative when approaching scientific questions. I would also like to thank my committee members, Dr. Jason Stajich for aid and guidance in bioinformatical aspects of my research, Dr. Richard Stouthamer for entomological guidance and support, and to Dr. Georgios Vidalalakis for showing me what issues, other than plant pathogens, grower operations face. You all helped me complete these research projects with an open mind not biased to one particular field of science. I would also like to thank our collaborators that helped contribute to this work including Christine Dodge, Paul F Rugman-Jones, Deena Husein, Matthew T Kasson, and Daniel Carrillo.

To those who have contributed and helped me with this work through the years including past lab members Danny Wang, Francis Na, Kameron Sugino Beth Peacock, Shannon Lynch thank you for making the transition into the lab an easy and comfortable process. To my undergraduate helpers and lab technicians Kevin Moreno, Diana Trannam, Dee Vega, Zhong Tan, Benedict Mamaril, Fabian Gonzalez, Divya Joshi, Edeli

Reyes, Emily Bossard, who all contributed to this work, it could not have been done without your committed time and efforts. A very special thanks to former lab member and fellow graduate student Dr. Joey Sal Mayorquin who taught me how to excel as a researcher and grew to become one of my great friends. Thank you for always being there for guidance and always making the long work days fun and enjoyable.

To my past advisor Dr. Kristin Bozak at Cal Poly Pomona I thank you for giving me and opportunity, believing in me, and pushing me to keep reaching higher. To PLPA students and former students that have always been there for advice and help and to Dr. Katherine Borkovich for reaching out and encouraging me to apply to the program. I wish to thank my family and friends for prolonged support through this long educational journey. And last but not least, to my loving wife Karissa who has been such a positive influence in my life and given me the greatest support through the years of graduate school

DEDICATION

This dissertation is dedicated to my wife Karissa, for believing in me when I didn't believe in myself and pushing me to achieve my academic goals and in memory of my grandmother Sadie Lopez who always believed in me and was so proud of my accomplishments.

ABSTRACT OF THE DISSERTATION

Diagnostic Detection, Biology, and Management of Invasive Shot Hole Borers and Fusarium Dieback in California

by

Joseph D Carrillo

Doctor of Philosophy, Graduate Program in Plant Pathology
University of California, Riverside, March 2019
Dr. Akif Eskalen, Co-Chairperson
Dr. Jason E. Stajich, Co-Chairperson

Since being detected in California in 2012, *Euwallacea whitfordiodendrus* and *E. kuroshio* commonly called shot hole borers (SHB) from Southeast Asia have been causing significant damage in multiple plant species across a range of habitats. Fusarium Dieback (FD) is a disease which results from repeated, local colonization of SHB and inoculation of their symbiotic ambrosia fungi (*Fusarium* spp., *Graphium* spp., *Paracremonium pembeum*) in woody tissues. Rapid molecular detection methods from infested host wood samples have been restricted to identification of the insect vector while management of this pest across their vast host range has been limited due to the cryptic nature of these pests. In addition, beetle-fungus interactions of the two *Euwallacea* spp. invading California and populations in Southeast Asia have not been explored. The work presented in this dissertation provides a validated real time quantitative polymerase chain reaction (PCR) assay which can detect all known plant

pathogenic fungi associated with *E. whitfordiodendrus* and *E. kuroshio* from pure cultures, avocado wood, and from female mycangia. Evidence from ground surveys indicate fungi from both species are detected together on some hosts in Orange and San Diego Counties. Investigation into beetle-fungal associations of *Euwallacea* spp. in Southeast Asia determined a beetle's association with their fungal symbionts to be non-exclusive and promiscuous. Both *MAT* genes were detected from the ambrosia fusaria clade (AFC) *Fusarium* spp. recovered from the beetles in native regions, while only one mating type (*MAT 1-1-1)* was determined present in California. We also demonstrated the ability of *E. whitfordiodendrus* and *E. kuroshio* to survive utilizing each other's ambrosia fungi, further indicating the relationship with their fungi can be promiscuous in invaded areas like California. Effective chemical management of the SHB pests in avocado groves was best achieved by use of the insecticide bifenthrin/zeta-cypermethrin.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

LIST OF TABLES

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

GENERAL INTRODUCTION

Bark and ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) are generally benign forest decomposers but some species cause significant economic damage on trees and timber (Beaver 1989). Bark beetles are typically phloeophagous, obtaining nutrients from the phloem tissue within the bark, while ambrosia beetles are known to be xylomycetophageous and their survival inside trees rely on mutualistic relationships with various symbiotic ambrosia fungi (Farrell et al. 2001). Sometimes the symbiotic fungi ambrosia beetles vector from host to host can be plant pathogens and the mutualistic relationship becomes destructive when introduced to new habitats from a native habitat such as Southeast Asia (Hulcr and Stelenski 2017). Such invasive pests, like *Xyloborus glabratus* with its pathogenic symbiont *Raffalea lauricola*, were introduced into southeastern United States around 2000 and has since caused the death of over a half a billion trees from the Lauraceae plant family (Hughes 2013). *Euwallacea* spp. are similarly equipped with plant pathogenic symbiotic ambrosia fungi, from various genera (Kasson et al 2013; Lynch et al. 2016; Na et al. 2018; O'Donnell et al. 2015;), and have been introduced to multiple areas of the United States including: California, Florida, Hawaii, as well as much of the mid-Atlantic and Southeastern United States (CABI 2015; Cognato et al. 2015; Eskalen et al. 2013; Na et al. 2018; O'Donnell et al. 2015; Rabaglia et al. 2006) and other parts of the world (CABI 2015; Paap et al. 2018; Stouthamer et al. 2017). Since being detected in California in 2012, the invasive shot hole borers (SHB) from Southeast Asia have been causing significant damage in multiple woody plant species (Eskalen et al. 2013). Fusarium Dieback (FD) is a disease resulting from repeated

local colonization of SHB and their symbiotic ambrosia fungi (*Fusarium* spp., *Graphium* spp., *Paracremonium pembeum*) in woody tissues which can obstruct water and mineral transport, resulting in dieback, wilt, and potentially mortality of the host tree (Eskalen et.al. 2013). The SHB-FD pest disease complex has affected over 300 species from 58 plant families including the agricultural commodity avocado (Eskalen et al. 2013). California presents a unique situation, being the only invaded region known to date in which two distinct *Euwallacea* spp. (Stouthamer et al. 2017) vectoring different plant pathogenic species of ambrosia fungi (Na et al. 2018) are causing FD throughout southern region. Currently, the SHB-FD pest-disease complex is spreading throughout southern California as far north as San Louis Obispo County and as far south as San Diego County; and a cause of great concern since there are no known effective treatments against these pests to manage the damage they are causing to susceptible plant species in established areas. The scope of the work presented here is focused on the invasion within California with emphasis on providing a reliable diagnostic for trees with FD, the nature of insect-fungal interactions and fungal mating populations in California as well as native Southeast Asian habitats, fungal symbiont fidelity for the two *Euwallacea* spp. present in California, and management of this pest in affected avocado groves

Fungal transmission and vector life cycle. The two *Euwallacea* spp. in California have since been named *E. whitfordiodendrus* (Schedl 1942) and *E. kuroshio* (Gomez and Hulcr 2018) which are associated with distinct symbiotic fungal pathogens that cause FD. *E. whitfordiodendrus* was first identified on avocado (Eskalen et al. 2012) and misidentified as *E. fornicatus* (Eichhoff 1868) commonly known as the tea shot hole borer (TSHB).

Since, *E. whitfordiodendrus* has been given the common name polyphagous shot hole borer (PSHB) and has been described to be associated with *F. euwallaceae* S. Freeman, Z. Mendel, T. Aoki & O'Donnell, in addition to *G. euwallaceae* M. Twizeyimana, S.C. Lynch & A. Eskalen, and *P. pembeum* S.C. Lynch & Eskalen within susceptible hosts (Eskalen et al. 2013; Mendel et al. 2012; Lynch et al. 2016). In 2014, *E. kuroshio* known commonly as the Kuroshio shot hole borer (KSHB), was found associated with *F. kuroshium* F. Na, J. D. Carrillo & A. Eskalen and *G. kuroshium* F. Na, J. D. Carrillo & A. Eskalen (Na et al. 2017) with no known *P. pembeum* recovered from *E. kuroshio* to date. The females are equipped with specialized organs located near their mandibles termed mycangia (Francke-Grosmann, 1956; Batra, 1963) which enable them to carry their symbiotic ambrosia fungi from host to host. The beetles initiate fungal inoculation when inseminated adult females bore into susceptible trees creating tunnels, or galleries, within the sapwood where they utilize their symbiotic ambrosia fungi as a food source and farm the fungi on plant tissue. Once the gallery is constructed and inoculation initiated, the female lays eggs which develop into adults feeding on the fungi present in the brood gallery. Their haplo-diploid inbred reproduction (arrhenotokous inbreeding) results in a predominantly female population which ensures a complete life cycle within one gallery without the need to seek a mate (Vega and Hofstetter 2015). Males are haploid, unable to fly, and typically remain in the gallery, while adult female offspring will emerge from the entry point of the gallery to inoculate and colonize the same or nearby host. The life cycle *in vitro* at 24°C is reported to be 22 days for *E. whitfordiodendrus* with an average of 32 females being produced each cycle (Cooperband et al. 2016). FD results as a combined

effect from multiple attacks and multiple fungal inoculations from females on the same host, blocking xylem nutrient and water transport (Eskalen et al. 2013, Hulcr and Stelinski 2017). All fungi associated with *E. whitfordiodendrus* and *E. kuroshio* have been reported as plant pathogens (Eskalen et al. 2012; Lynch et al. 2016; Na et al. 2018) with the AFC members thought to be the primary symbiotic ambrosia fungi (Kasson et al. 2013; O'Donnell et al. 2015) while *Graphium* spp. and *P. pembeum* appear to be auxiliary (Freeman et al. 2016).

It has been traditionally assumed that ambrosia beetles are mostly associated with a single dominant fungus (Kostocvik et al. 2015). However, it has been shown that they may feed on more than one species in fungal galleries (Batra 1966). Lateral transfer of fungal symbionts or "fungal sharing" has been reported from ambrosia beetles colonizing the same host (Carrillo et al. 2014, Hulcr and Cognato 2010) and in sympatric regions in Florida (Kostocvik et al. 2015). Initial research on AFC fungi associated with *Euwallacea* spp. cryptic species complex have been generally focused on invasive members (Eskalen et al. 2013; Kasson et al. 2013; Mendel et al. 2012; O'Donnell et al. 2015) and it has even been suggested that PSHB and TSHB are strictly obligate to their AFC fungi, *F. euwallaceae* and *F. ambrosium,* respectively (Freeman et al. 2013). Although promiscuous symbiosis has been reported in many other ambrosia beetle species in invaded areas like Florida (Carrillo et al. 2014; Kostocvik et al. 2015), the insect-fungal relationships between *Euwallacea* spp. and their symbiotic fungi has limited investigation within invaded areas and native habitats like Taiwan.

Host Range and Symptoms. Since its initial discovery within Los Angeles County in 2012, *E. whitfordiodendrus* has spread to several counties throughout southern California, including Orange, Riverside, San Bernardino, San Diego, and Ventura (https://ucanr.edu/sites/pshb/Map/), while *E. kuroshio* was initially identified in San Diego County but has since been detected in Orange, San Luis Obispo, and Santa Barbara Counties. The beetles can attack over three-hundered plant species from fifty-eight plant families (Eskalen et al. 2013) but reproductive hosts are most affected since these hosts support the full life cycle of the pests. Sixty-four plant species to date are reproductive hosts which includes native landscape species such as *Platanus racemosa* (California sycamore), native riparian species *Populus* spp., *Salix* spp., and the agricultural commodity *Persea americana* Mill (avocado) (https://ucanr.edu/sites/pshb/overview/SHB_Reproductive_Hosts/). In early stages of host infestation, signs of beetle attacks are visible as entry holes (~0.85mm diameter) and symptoms can be seen through various host responses to the boring activity of the beetle which include, gumming, sapping, staining, and sugar exudation (Eskalen et al. 2013). Although *E. whitfordiodendrus* and *E. kuroshio* are associated with different symbiotic ambrosia fungi, the hosts range of the two to date has been similar. The beetles are often misidentified for other common benign bark beetles and ambrosia beetles producing similar symptoms on various dead or dying trees. Although there are diagnostic assays to identify the beetles (Rugman-Jones and Stouthamer 2017) as well as AFC members (Short et al. 2017), currently there are no reliable diagnostic assays that target the *all* known fungal pathogens associated with the pests.

Economic impact. The impact of the disease can be severe in some areas, such as in urban landscapes, where *E. whitfordiodendrus* has infested 1/3 of California sycamore (*Platanus racemosa*) in Orange County public parks and caused the removal of 1262 trees, resulting in approximately $4 million USD in tree removal costs (OC Parks 2016). They have also been observed in riparian areas such as the Tijuana river valley in San Diego, which is dominated by *Salix* spp. and *Populus* spp., where *E. kuroshio* infestation levels are estimated to be greater than 53 percent in the area as of 2015 (Boland 2016). Within avocado groves, a recent survey found 80% of 18 surveyed groves were positive for beetle infestation in San Diego and Orange Counties, while in Ventura county 14 percent of 35 surveyed groves were found positive with the latter number expected to increase as the beetles spread northward (Lynch et al. 2018). This is a concern for the avocado industry since California is the largest producer of avocados grown in the United States and with over 50,000 bearing acres at an estimated value of 345,000,000 USD (California Avocado Commission 2018). Unfortunately, the effects of these pests on avocado production is currently unknown and management of the pest in groves is limited to only cultural management.

Current Management. General proposed methods to manage beetle populations include removal of infested wood, subsequent solarization or chipping, and in severe cases tree removal (Eatough-Jones and Paine 2015). However, this may not be practical in a native riparian habitat or avocado groves which are commonly grown on steep hills. The option of chemical management has been explored with some injectable and spray pesticide treatments including emamectin benzoate (insecticide), a combination of emamectin

benzoate and propiconazole (fungicide), and bifenthrin (insecticide) were found to significantly reduce new attacks from *E. whitfordiodendrus* in California sycamore (Mayorquin et al. 2018), as well as imidacloprid (insecticide) (Eatough-Jones et al. 2018) providing options for urban landscapes. Unfortunately, the previously determined effective chemicals are not currently registered for use on agricultural commodities such as avocado. Phytosanitation through pruning as a method of cultural management to reduce the number of colonized branches in a grove by removal of infested branches has been suggested (Mendel et al. 2017), however chemical management for PSHB and KSHB on avocado has not been thoroughly investigated.

Objectives. The work done here was aimed at investigating FD disease vectored by *E. whitfordiodendrus* and *E. kuroshio* invading California and providing methods for rapid diagnostics, information on the biology and interaction of *Euwallacea* spp. with their ambrosia fungi, and management strategies to control this pest in avocado groves. The objectives of the project include: (i) Development of a rapid diagnostic assay using quantitative Polymerase Chain Reaction (qPCR) to distinguish ambrosia fungi from both *E. whitfordiodendrus* and *E. kuroshio* infested wood samples; (ii) investigation into symbiotic ambrosia fungi fidelity with *Euwallacea* spp. in Taiwan as well as identifying mating types within the two AFC Fusaria populations in invaded areas and native populations; (iii) Determining if both *E. whitfordiodendrus* and *E. kuroshio* populations are exclusively associated with their symbiotic ambrosia fungi and (iv) investigate registered pesticides for chemical management to control these pests in avocado groves.

LITERATURE CITED

Batra, L.R., 1963. Ecology of ambrosia fungi and their dissemination by beetles. Transactions of the Kansas Academy of Science (1903-), 66(2), 213-236.

Batra, L.R., 1966. Ambrosia fungi: extent of specificity to ambrosia beetles. Science, 153(3732), 193-195.

Beaver, R.A., Sittichaya, W. and Liu, L.Y., 2014. A synopsis of the scolytine ambrosia beetles of Thailand (Coleoptera: Curculionidae: Scolytinae). Zootaxa, 3875(1), 1-82.

Boland, J.M., 2016. The impact of an invasive ambrosia beetle on the riparian habitats of the Tijuana River Valley, California. PeerJ, 4,.e2141.

CABI 2015. *Euwallacea fornicatus* Distribution Map. http://www.cabi.org/isc/datasheet/57163.

California Avocado Commission. 2018. Industry Statistical Data, 2016-2017. http://www.californiaavocadogrowers.com/industry/industry-statistical-data.

Carrillo, D., Duncan, R.E., Ploetz, J.N., Campbell, A.F., Ploetz, R.C. and Peña, J.E., 2014. Lateral transfer of a phytopathogenic symbiont among native and exotic ambrosia beetles. Plant Pathol., 63(1), 54-62.

Carrillo, D., Narvaez, T., Cossé, A.A., Stouthamer, R. and Cooperband, M., 2015. Attraction of *Euwallacea* nr. *fornicatus* (Coleoptera: Curculionidae: Scolytinae) to lures containing quercivorol. Fla. Entomol., 98(2), 780-782.

Cognato, A.I., Hoebeke, E.R., Kajimura, H. and Smith, S.M. 2015. History of the exotic ambrosia beetles *Euwallacea interjectus* and *Euwallacea validus* (Coleoptera: Curculionidae: Xyleborini) in the United States. Journal of economic entomology, *108*(3), 1129-1135.

Cooperband, M.F., Stouthamer, R., Carrillo, D., Eskalen, A., Thibault, T., Cossé, A.A., Castrillo, L.A., Vandenberg, J.D. and Rugman-Jones, P.F., 2016. Biology of two members of the *E uwallacea fornicatus* species complex (Coleoptera: Curculionidae: Scolytinae), recently invasive in the USA, reared on an ambrosia beetle artificial diet. *Agr*. Forest Entomol., 18(3), 223-237.

Eskalen, A., Gonzalez, A., Wang, D.H., Twizeyimana, M., Mayorquin, J.S. and Lynch, S.C., 2012. First report of a Fusarium sp. and its vector tea shot hole borer (Euwallacea fornicatus) causing Fusarium dieback on avocado in California. Plant Dis. *96*(7), 1070-1070.

Eskalen, A., Stouthamer, R., Lynch, S. C., Rugman-Jones, P. F., Twizeyimana, M., Gonzalez, A., & Thibault, T. 2013. Host Range of Fusarium Dieback and Its Ambrosia Beetle (Coleoptera: Scolytinae) Vector in Southern California. Plant Dis., 97(7), 938–951.

Farrell, B.D., Sequeira, A.S., O'Meara, B.C., Normark, B.B., Chung, J.H. and Jordal, B.H., 2001. The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). Evolution, 55(10), 2011-2027.

Franke-Grosmann, H. 1956. Hautdrüsen als Träger der Pilzsymbiose bei Ambrosiakäfern. Z. Morph. u. Ökol. Tiere, 45**,** 275-308

Freeman, S., Sharon, M., Maymon, M., Mendel, Z., Protasov, A., Aoki, T., Eskalen, A., O'Donnell, K. 2013. *Fusarium euwallaceae* sp. nov.--a symbiotic fungus of *Euwallacea sp*., an invasive ambrosia beetle in Israel and California. Mycologia, 105(6), 1595–1606.

Freeman, S., Sharon, M., Dori-Bachash, M., Maymon, M., Belausov, E., Maoz, Y., Margalit, O., Protasov, A. and Mendel, Z., 2016. Symbiotic association of three fungal species throughout the life cycle of the ambrosia beetle *Euwallacea* nr. *fornicatus*. Symbiosis, 68(1-3), 115-128.

Hughes MA. 2013. The evaluation of natural resistance to laurel wilt disease in redbay (*Persea borbonia*). PhD Thesis, Univ. Florida, Gainesville

Hulcr, J., & Cognato, A. I. 2010. Repeated evolution of crop theft in fungus-farming ambrosia beetles. Evolution, 64(11), 3205–3212.

Hulcr, J., & Stelinski, L. L. 2017. The Ambrosia Symbiosis: From Evolutionary Ecology to Practical Management. Annu. Rev. Entomol., 62, 285-303.

Kasson, M. T., O'Donnell, K., Rooney, A. P., Sink, S., Ploetz, R. C., Ploetz, J. N., Konkol, J. L., Carrillo, D., Freeman, S., Mendel, Z., Smith, J. A., Black, A. W., Hulcr, J., Bateman, C., Stefkova, K., Campbell, P. R., Geering, A. D. W., Dann, E. K., Eskalen, A., Mohotti, K., Short, D. P. G., Aoki, T., Fenstermacher, K. A., Davis, D. D., Geiser, D. M. 2013. An inordinate fondness for *Fusarium*: Phylogenetic diversity of fusaria cultivated by ambrosia beetles in the genus *Euwallacea* on avocado and other plant hosts. Fungal Genet. Biol., 56, 147–157.

Kostovcik, M., Bateman, C.C., Kolarik, M., Stelinski, L.L., Jordal, B.H. and Hulcr, J., 2015. The ambrosia symbiosis is specific in some species and promiscuous in others: evidence from community pyrosequencing. ISME J, 9(1), 126.

Lynch, S. C., Twizeyimana, M., Mayorquin, J. S., Wang, D. H., Na, F., Kayim, M., ... & Hulcr, J. 2016. Identification, pathogenicity and abundance of *Paracremonium pembeum* sp. nov. and *Graphium euwallaceae* sp. nov.—two newly discovered mycangial associates of the polyphagous shot hole borer (*Euwallacea sp*.) in California. Mycologia, 108(2), 313-329.

Lynch, S.C., Carrillo, J.D., Stouthamer, R., Eskalen, A. 2018. Severity of Fusarium Dieback – Shot Hole Borers Analyzed. From The Grove. Vol.8, No 4. 46-50

Mayorquin, J.S., Carrillo, J.D., Twizeyimana, M., Peacock, B.B., Sugino, K.Y., Na, F., Wang, D.H., Kabashima, J.N. and Eskalen, A., 2018. Chemical Management of Invasive Shot Hole Borer and Fusarium Dieback in California Sycamore (*Platanus racemosa*) in Southern California. Plant Dis., 102(7), 1307-1315.

Mendel, Z., Protasov, A., Sharon, M., Zveibil, A., Yehuda, S.B., O'Donnell, K., Rabaglia, R., Wysoki, M. and Freeman, S., 2012. An Asian ambrosia beetle *Euwallacea fornicatus* and its novel symbiotic fungus *Fusarium* sp. pose a serious threat to the Israeli avocado industry. Phytoparasitica, 40(3), 235-238.

Na, F., Carrillo, J.D., Mayorquin, J.S., Ndinga-Muniania, C., Stajich, J.E., Stouthamer, R., Huang, Y.T., Lin, Y.T., Chen, C.Y. and Eskalen, A., 2018. Two novel fungal symbionts *Fusarium kuroshium* sp. nov. and *Graphium kuroshium* sp. nov. of Kuroshio shot hole borer (*Euwallacea* sp. nr. *fornicatus*) cause Fusarium dieback on woody host species in California. Plant dis., 102(6), 1154-1164.

OC Parks 2016. Shot Hole Borer: Managing the Invasive Beetle. https://oc-parks-gis.maps.arcgis.com/apps/Cascade/index.html?appid=680fd0c9e73f4857a8477791f7ee796f.

O'Donnell, K., Sink, S., Libeskind-Hadas, R., Hulcr, J., Kasson, M. T., Ploetz, R. C., Konkol, J. L., Ploetz, J. N., Carrillo, D., Campbell, A., Duncan, R. E., Liyanage, P. N., Eskalen, A., Na, F., Geiser, D. M., Bateman, C., Freeman, S., Mendel, Z., Sharon, M., Aoki, T., Crossé, A. A., Rooney, A. P. 2015. Discordant phylogenies suggest repeated host shifts in the *Fusarium-Euwallacea* ambrosia beetle mutualism. Fungal Genet. Biol., 82, 277–290.

O'Donnell, K., Libeskind-Hadas, R., Hulcr, J., Bateman, C., Kasson, M.T., Ploetz, R.C., Konkol, J.L., Ploetz, J.N., Carrillo, D., Campbell, A. and Duncan, R.E., 2016. Invasive Asian *Fusarium–Euwallacea* ambrosia beetle mutualists pose a serious threat to forests, urban landscapes and the avocado industry. Phytoparasitica, 44(4), 435-442.

Paap, T., De Beer, Z.W., Migliorini, D., Nel, W.J. and Wingfield, M.J., 2018. The polyphagous shot hole borer (PSHB) and its fungal symbiont *Fusarium euwallaceae*: a new invasion in South Africa. Australas. Plant Path., 47(2), 231-237.

Rugman-Jones, P.F. and Stouthamer, R., 2017. High-resolution melt analysis without DNA extraction affords rapid genotype resolution and species identification. Mol. Ecol. Resour., 17(4), 598-607.

Short, D.P., O'Donnell, K., Stajich, J.E., Hulcr, J., Kijimoto, T., Berger, M.C., Macias, A.M., Spahr, E.J., Bateman, C.C., Eskalen, A. and Lynch, S.C., 2017. PCR Multiplexes Discriminate *Fusarium* Symbionts of Invasive *Euwallacea* Ambrosia Beetles that Inflict Damage on Numerous Tree Species Throughout the United States. Plant Dis., 101(1), 233-240.

Stouthamer, R., Rugman-Jones, P., Thu, P.Q., Eskalen, A., Thibault, T., Hulcr, J., Wang, L.J., Jordal, B.H., Chen, C.Y., Cooperband, M. and Lin, C.S., 2017. Tracing the origin of a cryptic invader: phylogeography of the *Euwallacea fornicatus* (C oleoptera: Curculionidae: Scolytinae) species complex. Agricultural and forest entomology, 19(4), 366-375.

Vega, F.E. and Hofstetter, R.W. eds., 2015. Bark beetles: biology and ecology of native and invasive species. Academic Press.

CHAPTER I. Probe-based multiplex real-time PCR as a diagnostic tool to distinguish distinct fungal symbionts associated with *Euwallacea* spp. in California

ABSTRACT

California has been invaded by two distinct *Euwallacea* spp. that vector unique plant pathogenic symbiotic fungi on multiple hosts and cause Fusarium Dieback (FD). The objective of this study was to develop multiplex real time quantitative PCR assays using hydrolysis probes targeting the β-tubulin gene to detect, distinguish, and quantify fungi associated with polyphagous shot hole borer (PSHB; *Fusarium euwallaceae, Graphium euwallaceae,* and *Paracremonium pembeum*) and Kuroshio shot hole borer (KSHB; *F. kuroshium* and *G. kuroshium*), respectively, from various sample types. Absolute quantification reaction efficiencies ranged from 88.2 to 104.3 percent with a coefficient of determination greater than 0.992 and a limit of detection (LoD) of 100 copies μl^{-1} for all targets across both assays. Qualitative detection using the real-time assays on artificially inoculated avocado shoot extracts showed more sensitivity compared to conventional fungal isolation from wood. All symbiotic fungi, except *P. pembeum*, from PSHB and KSHB female heads were detectable and quantified. Field samples from symptomatic *Platanus racemosa, Populus* spp., and *Salix* spp. across 17/26 city parks were positively identified as PSHB and KSHB and found both occurring together on five trees from three different park locations. The molecular assays presented here can be utilized to accurately identify fungi associated with these invasive pests in California.

INTRODUCTION

Bark and wood-boring weevils from the Scolytidae sub-family include more than 6000 species that are of considerable economic importance in both temperate and tropical forests (Beaver et al. 2014). The Xyloborini tribe within this sub-family contain xylomycetophagous ambrosia beetles that are currently invading multiple locations in the world (Beaver et al. 2014; CABI 2015; O'Donnell et al. 2016). Ambrosia beetles are known to be associated with fungal and bacterial organisms (Beaver et al. 2014), with the fungal symbionts sometimes being phytopathogenic (Eskalen et al. 2013; Hulcr and Dunn 2011; Mendel et al. 2012; Ploetz et al. 2013). Fusarium Dieback (FD) is a vascular disease of hardwood trees in southern California and is part of a highly invasive pest-disease complex threatening the avocado industry, urban forests and native wildlands. There have been two separate invasions of exotic ambrosia beetles in California commonly called polyphagous shot hole borer (PSHB, Eskalen et al. 2013) and Kuroshio shot hole borer (KSHB, Stouthamer et al. 2017) both carrying similar but distinct suites of phytopathogenic fungi (Freeman et al. 2013; Lynch et al. 2016; Na et al. 2018). The beetles have a wide host range of over 300 plant species that are attacked, with sixty-four of those being suitable hosts for beetle reproduction (https://ucanr.edu/sites/pshb/overview/Hosts), and is a great concern for the region since there are limited strategies available for management of the pests.

Reproductive hosts are at more risk (Eskalen et al. 2013) and the progression of FD becomes severe when beetles bore into the xylem then continue to proliferate and accumulate on the host (Hulcr and Stelinski 2017). In early stages of host infestation,

signs of beetle attacks are visible as entry holes (~0.85mm diameter) and symptoms can be seen through various host responses to the boring activity of the beetle which include, gumming, sapping, staining, and sugar exudation (specific to avocado, aka sugar volcano) (Eskalen et al. 2013). The impact of the disease can be severe in some areas such as in urban landscape where PSHB infested 1/3 of California sycamore (*Platanus racemosa*) in Orange County public parks and caused the removal of 1262 trees, resulting in approximately $4 million USD in tree removal costs (OC Parks 2016). In riparian areas such as the Tijuana river valley, which is dominated by *Salix* spp. and *Populus* spp., KSHB infestation levels are estimated to be greater than 53 percent in the conservation area as of 2015 (Boland 2016). The beetles are often misidentified for other common benign bark beetles and ambrosia beetles producing similar symptoms on various dead or dying trees. Although beetles can be recovered from the host for identification, they are usually difficult to obtain since they are generally protected deeper in the wood (25-100 mm) of the tree or not present at all, depending on the age of the beetle gallery. The beetles tend to inoculate their symbiotic fungi as they initiate boring gallery tunnels in to tree, however, the fungi will still grow in the plant tissues (Hofstetter et al. 2015) whether the beetle is present or not. Therefore, targeting the symbiotic fungi associated with both PSHB and KSHB can provide accurate detection of these pest-disease complexes in the variety of hosts they colonize in California.

PSHB and KSHB are morphologically indistinguishable and have been recently reclassified as *Euwallacea whitfordiodendrus* (Schedl 1942) and *E. kuroshio* (Gomez and Hulcr 2018). Means to detect and distinguish these two ambrosia beetles has been

described using conventional PCR and Sanger sequencing of the COI (cytochrome oxidase I) locus (O'Donnell et al. 2015; Stouthamer et al. 2017) as well as a rapid high-resolution melt curve (HRMC) analysis using real time PCR targeting the region (Rugman-Jones and Stouthamer, 2017). The phytopathogenic fungi associated with the two cryptic species also differ in this invaded region; PSHB has been described to be associated with *Fusarium euwallaceae* S. Freeman, Z. Mendel, T. Aoki & O'Donnell, *Graphium euwallaceae* M. Twizeyimana, S.C. Lynch & A. Eskalen, and *Paracremonium pembeum* S.C. Lynch & Eskalen, while KSHB is associated with *Fusarium kuroshium* F. Na, J. D. Carrillo & A. Eskalen and *Graphium kuroshium* F. Na, J. D. Carrillo & A. Eskalen. There have been efforts to develop conventional multiplexed conventional PCR assays to target the *Fusarium* spp. associated with *Euwallacea* spp. (Short et al. 2017), also known as AFC fusaria (Kasson et al. 2013), but no efforts have been made to detect *all* the known fungi associated with PSHB and KSHB, respectively, from recovered samples.

Several polymerase chain reaction (PCR) based molecular methods have been developed for detection of plant pathogens and among these methods, hydrolysis probe-based quantitative real-time PCR (qPCR) assays are the among the most sensitive (Biassoni and Raso, 2016; Palacio-Bielsa, Cambra, and López, 2009; Postollec et al. 2009). Internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) are some of most commonly used targets for designing PCR assays to detect and identify fungal pathogens (Glass and Donaldson 1995; Schoch et al. 2012; White et al. 1990). Ribosomal DNA can be present in more than 100 copies per genome and has been reported to

increase detection sensitivity (Tellenbach et al. 2010). However, in previous studies (Kasson et al. 2013; Na et al. 2018; O'Donnell et al. 2015) the ITS region of AFC fusaria associated with *Euwallacea* spp. was not as informative in distinguishing species due to lack of polymorphic sites compared to other loci such as translation elongation factor 1α (TEF1- α), DNA-directed RNA polymerase II largest (RPB1), and second largest subunit (RPB2). For example, *F. euwallaceae* from PSHB and *F. kuroshium* from KSHB were previously found to be completely identical at the ITS region (Na et al. 2018). A target sequence that consists of a single-copy gene per haploid genome and has more polymorphic regions, such as the β-tubulin gene, has been shown to provide a more reliable quantification of fungal DNA from different samples types in multiplex qPCR reaction conditions (Atallah et al. 2007, Billones-Baaijens et al. 2018; Pouzoulet et al. 2013). The ability to detect and quantify fungal DNA using qPCR has been previously developed and applied to detect and quantify plant pathogens from plant tissue (Li et al. 2006; L. Li et al. 2009; Oliveira et al. 2002; Qu et al. 2011). Although the real-time PCR technology has been applied to detect a variety of plant pathogens affecting woody tree species (Bodles et al, 2006; Chandelier et al. 2018; Heller and Keith, 2018; Osman and Rowhani, 2006; Pouzoulet et al. 2013), none have been developed to target fungal pathogens vectored by invasive *Euwallacea* spp. that are currently causing damage in a wide range of hosts.

The objectives of this study were to (i) develop unique sets of species specific oligonucleotides and hydrolysis probes to target and detect *F. euwallaceae*, *G. euwallaceae,* and *P. pembeum* (PSHB) as well as *F. kuroshium* and *G. kuroshium*

(KSHB); (ii) optimize multiplex qPCR assays for each complex to qualitatively identify the fungal targets across multiple sample types including DNA extracts from infested wood, female beetle heads, and pure fungal cultures recovered from various reproductive hosts; (iii) quantify fungal targets from artificially inoculated avocado wood and from lab-reared PSHB and KSHB female heads; and (iv) elucidate the distribution of both complexes from wood samples of cottonwood, sycamore, and willow collected from field surveys in sympatric areas.

MATERIALS AND METHODS

Fungal isolates. Multiple isolates of fungal species (*F. euwallaceae, F. kuroshium, G. euwallaceae, G. kuroshium,* and *P. pembeum*) obtained from previous studies (Eskalen et al. 2013; Na et al. 2018; Lynch et al. 2016) were used in this study from a maintained culture collection from the Eskalen lab at the University of California, Riverside. In addition, isolates of *F. solani*, *F. oxysporum*, *F. brasiliense, Alternaria alternata, Clonostachys rosea, Colletotrichum gloeosporoides, Neofusicoccum parvum,* and *Penicillium expansum* used in this study (Table 1.1) were included to test PSHB-FD and KSHB-FD assays for specificity. Fungal isolates recovered from beetles used in this study were obtained from the heads of beetles using methods described by Lynch et al. 2016 and isolates recovered from galleries in wood followed methods described by Eskalen et al. 2013 and Na et al. 2018. All other isolates were provided by the Agricultural Research Service culture collection under permit (USDA- P526P-17-02585). All isolates used in this study were maintained on potato dextrose agar (PDA; BD Difco,

Sparks, MD, USA) amended with 0.01% tetracycline hydrochloride grown at 25°C for no longer than two weeks.

DNA extractions. For DNA extraction of fungi from pure cultures, mycelium was harvested from fungal isolates maintained on PDA from petri dishes once fully colonized and placed into sterile 1.5 mL microcentrifuge tubes containing 25µl of AP1 buffer (Qiagen, Hilden, Germany) then frozen at -80°C and macerated with a plastic pestle (Thermo Fisher Scientific, Pittsburgh, PA, USA). Once the tissue was macerated, the DNeasy plant mini kit (Qiagen, Hilden, Germany) manufacturer's protocol was used to extract DNA from the samples. All samples were suspended in 50µl AE elution buffer and DNA concentration was quantified using Nanodrop 2000c (Thermo Fisher Scientific, Pittsburgh, PA, USA) and diluted to a concentration of 10 ng/µl.

For DNA extraction from symptomatic woody tissue, samples were first lyophilized with a bench top freezedryer (Labconco FreeZone 2.5, Kansas City, MO, USA) at -50°C under 0.01 mbar vacuum (Labconco Rotary Vane Vacuum Pump, 195 LPM, 230V, Kansas City, MO, USA) for 24 hours. Wood samples were then transferred to sterile 2 ml stainless steel metal microcentrifuge tubes along with 2 sterile stainless-steel beads (2 mm diameter) and a sterile cotton plug then re-frozen at -80°C for 30 min. Once frozen, samples were capped with a sterile impact resistant plastic plug and bead beaten for 30 sec at 4.0 m/s using a FastPrep (MP Biomedicals, Solon, OH). Resulting wood dust was used for DNA extraction using DNeasy plant mini kit manufacturer protocol (Qiagen, Hilden, Germany). All samples were suspended in 50µl AE elution

buffer and DNA concentration was quantified using Nanodrop 2000c (Thermo Fisher Scientific, Pittsburgh, PA, USA)

For DNA extraction from female beetles, samples were surface sterilized for 10 s using 1mL 70% ethanol followed by two water washes using 1mL sterile deionized water. Once washed, the heads of the beetles were separated from the rest of the body and placed into sterile 1.5 mL Eppendorf tubes, and frozen at -80°C for 30 min then macerated with a plastic pestle (Thermo Fisher Scientific, Pittsburgh, PA, USA). Once the tissue was macerated, the DNeasy plant mini kit (Qiagen, Hilden, Germany) was used to extract DNA from the samples. All samples were suspended in 50µl AE elution buffer and DNA concentration was quantified using Nanodrop 2000c (Thermo Fisher Scientific, Pittsburgh, PA, USA).

Primer and probe design. Sequences from target *Fusarium* spp. were obtained from available genomes (GenBank Accessions: NHTE00000000, NKUJ00000000, NIZV00000000, NKCL00000000, NKCK00000000, NKCJ00000000, NKCI00000000) and checked for specificity by utilizing the FASTA program (Pearson 2016) against available genomes using the fasta36 tool. *Graphium* spp. and *Paracremonium* spp. *TUB2* regions were amplified using conventional PCR with Bt1a and Bt1b primer sets (Glass and Donaldson 1995). Products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) then sequenced in both directions (Sanger ABI 3730) at the Institute for Integrative Genome Biology University of California-Riverside with corresponding primers used for PCR. Species specific primer sets and Primetime® hydrolysis probes were designed for each target species by aligning sequences of targets with sequences

obtained from GenBank (Table 1.1) from closely related members for each genus with Clustal X (Thompson *et al.,* 1997) to determine the presence of polymorphic regions in the β-tubulin (*TUB2*) gene. Polymorphic regions in the 4th intronic region of *TUB2* gene were selected and melting temperatures and secondary structures of generated oligonucleotides were evaluated using Primer3 (v. 0.4.0, Koressaar and Remm, 2007; Untergasser et al. 2012). Each primer set was designed to have at least one single nucleotide polymorphism (SNP) with one targeted on the 3' end of the oligonucleotide sequence. Hydrolysis probes were designed with 5 SNPs for target *Fusarium* spp. and 3 SNPs for target *Graphium* spp. between the respective species of interest (Table 1.2). Candidate oligonucleotide sequences were also checked for specificity by using basic local alignment search tool (BLAST) (Altschul et al. 1990). Separate assays for the two invasive pest disease complexes PSHB-FD and KSHB-FD in California were developed using 5 different flourophores selected for species specific hydrolysis probes targeting fungi from the PSHB-FD complex including FAM (*F. euwallaceae*), Texas 615 (*G. euwallaceae*), Cy5.5™ (*P. pembeum*), and HEX (*F. kuroshium*) and Cy5™ (*G. kuroshium*) for the KSHB-FD complex. All hydrolysis probes had 5' fluorophore and 3' Iowa Black™ dark quencher modifications and only FAM and HEX hydrolysis probes had internal ZEN™ quenchers; all were synthesized by Integrated DNA Technologies (IDT, San Diego, CA, USA). The oligonucleotide sequences, product length, and melting temperatures can be seen in Table 1.2.

Multiplexed real-time PCR reaction, optimization, and validation. The multiplexed PCR reactions were carried out in 25µl reactions using 1x iQ Supermix (Bio-Rad,

Hercules, CA, USA), 200nm of each primer set, 100nm of each hydrolysis probe, and 1µl DNA template or nuclease free water (Thermo Fisher Scientific, Pittsburgh, PA, USA) for no-template controls (NTC). All PCR amplifications were done using a 2-step protocol: 94°C for 3 m followed by 33 cycles of denaturation at 94°C for 15 s followed by annealing/extension at 60°C for 45 s with plate readings taking place after the annealing extension step in real-time runs. All qPCR reactions were done in triplicate using Hardshell® 96 well PCR plates (Bio-Rad, Hercules, CA, USA) or 0.2ml 8 tube low profile strips (Bio-Rad, Hercules, CA, USA) compatible with CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and analyzed using CFX manager (v.3.1, Bio-Rad, Hercules, CA, USA).

The optimal multiplexed annealing/extension conditions for all primer sets was determined to be 60°C using conventional PCR thermal gradient option with a MyCycler® (Bio-Rad, Hercules, CA, USA) and end-point analysis of amplified products were separated by gel electrophoresis in 2.5% agarose gel with 0.5x Tris-boric acid-EDTA buffer, stained with SYBR Green (Invitrogen, Carlsbad, CA), and visualized under UV light. Species-specific products were validated by using DNA from type specimens (Table 1.1) of the five target species and evaluating products based on single bands without significant primer dimer as well as correct amplicon size. Synthesized dsDNA of the each of the 5 species target region (gBlocks, IDT, San Diego, CA, USA) were for used for both generation of standard curves and positive controls for non-quantitative experiments. Lyophilized samples or the dsDNA gBlocks were suspended in nuclease free water (Thermo Fisher Scientific, Pittsburgh, PA, USA) and DNA

concentration quantified using Nanodrop 2000c (Thermo Fisher Scientific, Pittsburgh, PA, USA). The number of copies/µl were calculated using the following equation provided by the manufacturer:

$$(C)\ (M)\ (1 \times 10^{-15}\ \text{mol/fmol})\ (\text{Avogadro's number}) = \textit{copy number}/\mu L$$

Where *C* is the current concentration of the gBlocks Gene Fragment in ng/µl, and *M* is the molecular weight in fmol/ng, as provided from the manufacturer. The gBlocks were then diluted to a concentration of $1x10^6$ copies/µl for standard curve generation and diluted 10-fold to a concentration of 1 copies/µl. Limit of detection (LoD) for each assay was determined following the MIQE guidelines (Bustin et al. 2009) which is the number of copies required to reproducible detection and quantification across experimental assays respectively. Quantification cycles (Cq) values were generated from CFX manager (v.3.1, Bio-Rad, Hercules, CA, USA) with the Cq determination set to single threshold at a user defined 30 RFU baseline threshold value. Singleplex reaction Cq values from each species-specific primer/probe set to multiplex reaction Cq values for either PSHB or KSHB assays at a high concentration of $1x10^6$ copies/µl gBlocks and a the LoD concentration of $1x10^2$ copies/ µl (Table 1.3). Intra-assay and Inter-assay Cq values across six experimental plates were also analyzed with the same experimental parameters mentioned above (Table 1.4). For PSHB assays, gBlocks dsDNA standards were mixed at a 1:1:1 ratio/reaction for each target species (*F. euwallaceae/G. euwallaceae/P. pembeum*) of gBlocks suspensions and for KSHB assays, gBlocks standards were mixed at a 1:1 ratio/reaction for each target species (*F. kuroshium/G. kuroshium*) of gBlocks suspensions. For all quantification experiments, a total of five multiplexed standards were

used per assay; ranging from a high concentration of $1x10^6$ copies/µl diluted 10-fold to the LoD of 100 copies/µl. Multiplex assays were also tested for specificity to their target species from testing DNA extracts (10 ng/ul) from non-target species (Table 1.1) while using DNA extracts from type isolates as positive controls individually as well as a mixed reaction of type isolates (Table 1.1).

Detection from inoculated wood with target fungal species. Avocado stems from 2-year old "Zutano" seedlings were used for controlled inoculation of the target fungal species: *F. euwallaceae, F. kuroshium, G. euwallaceae, G. kuroshium,* and *P. pembeum*. The cuttings (20 cm long) were surface sterilized by soaking in a 10% household bleach solution for 10 minutes then rinsed with deionized water and allowed to dry. Although the use of a fungal plug of colonized agar is reported in other studies (Lynch et al. 2016; Pouzoulet et al. 2013), we decided to mimic the inoculation of these fungi by the vector by drilling small holes and depositing individual and mixed spore suspensions in combinations that exist in the field, as well as two mixed inoculations not found in the field to date (*Fusarium* spp. mix and *Graphium* spp. mix). Fungal spore suspensions were prepared from isolates of *F. euwallaceae, F. kuroshium, G. euwallaceae, G. kuroshium,* and *P. pembeum* (Table 1.5 and 1.6) by scraping one-week old sporulating cultures grown on PDA with a sterile inoculating loop and streaking onto fresh PDA media plates amended with 0.01% tetracycline hydrochloride grown at 25°C for three days. Five milliliters of sterile distilled water was added to each plate and agitated with sterile bent glass rods to release the spores into suspension before passing through a layer of sterile Kimwipe® to remove large mycelial fragments. All spore suspensions were quantified

using a Bright-Line hemocytometer (Hausser Scientific, Horsham, PA, USA) and diluted to $1x10^6$ spores/ml. To inoculate the shoots, five wounds were made per shoot with two replicate shoots per fungal species using a rotary tool (Dremel 7700 Series, Racine, WI, USA) with a flamed 7114 diamond wheel point attachment (Dremel, Racine, WI, USA). Ten microliters of spore suspensions and mixed combination treatments (Tables 1.5 and 1.6) were pipetted in the wounds and wrapped with parafilm for a total of 13 treatments, including a sterile water treated control, then placed in plastic containers elevated on plastic lighting diffusers lined with paper towels soaked with deionized water to maintain humidity. Shoots were destructively sampled after four weeks and 1 cm x 1 cm wood pieces were sampled from the discolored margins then plated on PDA amended with 0.01% tetracycline hydrochloride to recover the inoculated fungi. Remaining discolored wood (~200 mg) was weighed and frozen at -80°C. DNA extracts from the wood were used in quantification experiments for both PSHB and KSHB assays in triplicate and DNA concentrations were normalized to 10 ng per reaction as well as isolated on PDA amended with 0.01% tetracycline to compare recovery from conventional isolation methods (Table 1.5). Controls for the experimental plates included a negative control (extract from water treated wood) and a no-template control (NTC). The SPUD test (Nolan et al. 2006) was also performed to verify that the non-inoculated plant extract did not inhibit the PCR reaction significantly by spiking *F. euwallaceae, F. kuroshium, G. euwallaceae, G. kuroshium,* and *P. pembeum* isolate DNA templates at 10 ng per reaction with an equal concentration of avocado DNA extract.

Quantification of fungal symbionts from the beetle mycangia. Ten adult female beetles of each PSHB and KSHB were obtained from lab reared sawdust media colonies maintained with methods similar to Cooperband et al. 2016. For quantification experiments, DNA extracts from beetle heads were normalized to 10 ng per reaction and tested in both PSHB and KSHB assays in triplicate.

Field sampling. Twenty-six locations in Riverside (6), Orange (15), and San Diego (5) counties were surveyed for SHB attacks and samples were collected from *Platanus racemosa* (278), *Populus* spp. (59), *Salix* spp. (11) if visible symptoms of frass, sapping, and/or gumming were present with visible entry holes as described in Eskalen et al. (2013). These host trees were chosen since these hosts are favored by this pest and display obvious combinations of symptoms described above. Trees exhibiting symptoms were sampled in five different areas per tree with SHB entry holes visible and were collected by cutting an approximate 1 cm x 1 cm x 1 cm cube around the entry hole with a clean Buck knife (Buckknife, Post Falls, ID, USA) to remove the infested tissue as well as the beetle if present. The knife was cleaned with 95% ethanol via spray bottle after each sample. Once samples were collected, they were deposited in 1.5 ml microcentrifuge tubes, labeled, and placed in a cooler then transported back to the lab (CDFA plant pest permit #2887) for storage at 4°C until processing. Wood samples were surface sterilized by dipping the wood sample into 95% ethanol, flamed, then discolored margins were dissected with sterile tools near the entry holes and plated on PDA amended with 0.01% tetracycline hydrochloride. The remaining sample was placed in a sterile 1.5 ml microcentrifuge tube and frozen at -80°C. The isolation plates were

incubated at 28°C for at least three days. The fungal colonies used in this trial were selected based on their morphology described by Freeman et al. 2013; Lynch et al. 2016; Na et al. 2018). Fungal colonies of interest were sampled with a sterile toothpick and placed in a 0.5 ml microfuge tube with 25 µl sterile water to make a spore suspension. Resultant spore suspensions were used as a template for both PSHB and KSHB assays for downstream qualitative identification. All trees GPS locations as well as sample information was recorded using custom built forms on the iForm application (Zurion Software Inc., Herndon, VA, USA). All maps were created using R (version 3.4.3, Team, 2013) and the "leaflet" package (Cheng et al. 2017).

Statistical analyses. All statistical analyses were performed using R (version 3.4.3, Team, 2013). Analysis of Cq values from singleplex/multiplex and intra-assay/inter-assay comparisons were done using the "plyr" package (Wickham, 2011) to calculate means, standard deviation, standard error, as well as coefficient of variation (CV) values. Quantitative experiments were analyzed using estimated starting quantities across all treatments with the data separated by target species. Starting Quantities (SQ) from the samples were calculated from a standard curve run within each experimental plate run. Regression analysis using log transformed SQ values from inoculation treatments was performed by target species (fluorophore) (Table 1.6). Due to the over-dispersion of the data as the variance exceeds the mean, confirmed using the *dispersiontest* function from the package "AER" (Kleiber et al. 2018), regression analysis using a generalized linear model with a negative binomial link function was performed using the "MASS" package (Venables and Ripley, 2002). Detached shoot inoculation post-hoc analysis was done by

performing an all pairwise Tukey's test for the comparison across all responses to treatments by target. PSHB/KSHB female heads post-hoc analysis was done by constructing a contrast matrix in order to compare all responses to target species by biological sample (PSHB or KSHB) of interest then by using the "multcomp" package (Holthorn et al. 2008) with link function *glht* for comparison of all target species to each other based on the matrix. All bar plots were generated using "ggplot2" (Wickham, 2016). Statistics for the field survey recovery frequencies were also done using logistic regression measuring the frequency of recovery by plant species and recovered fungus genera using the binomial link function.

RESULTS

Primer/probe design and validation. The development of the species-specific primers for fungi associated with PSHB and KSHB were based on the nucleotide sequence alignment of the β-tubulin (*TUB2*) gene sequences as the ITS and translation elongation factor 1-α (TEF1-α) genes had few polymorphic regions to exploit within targeted genera and the specific species to be distinguished. *In silico* results from BLAST indicated most species-specific primer sets and probes had <100% matches to existing accessions other than the targets, except for *Fusarium* spp. primer sets where one had a 100% match to *F. solani*, however the probes were all found to be specific with <100% matches to existing accessions other than the targets. It should be noted that *Graphium* spp. and *Paracremonium* spp. have limited accessions submitted to GenBank. Preliminary primer optimization using conventional PCR showed the optimum annealing/extension temperature for all species-specific primer sets of interest was 60°C which amplified

products of expected sizes listed in Table 1.2 without the presence of primer dimers after agarose gel electrophoresis. No PCR products were observed when non-target DNA extracts (Table 1.2) were tested with species-specific primer sets.

Development of multiplex real-time PCR assays. Initially we intended to develop a multiplex assay that would allow for the detection of all fungi associated with PSHB and KSHB simultaneously in one reaction, however, we had issues with significant differences in detection (Cq values) using DNA from all five targets present in multiplex vs singleplex reactions (data not shown). Therefore, we decided to develop two assays: a PSHB assay and a KSHB assay which did not show significantly higher Cq values ($P > 0.05$) in multiplex assays compared to singleplex assays (Table 1.3). Inter-assay and Intra-assay Cq values were not significantly different across the experimental plates tested (Table 1.4). Non-template control (NTC) values for target *Fusarium* spp. produced a signal after 33 cycles, therefore cycling conditions should not go longer than 33 cycles. Reaction efficiencies ranged from 88.2% to 104.3% with $R^2 \geq 0.992$ were obtained across all targets based on the slopes generated from standard curves of synthesized dsDNA suspensions (gBlocks) covering a dynamic range of $1x10^6$ to $1x10^2$ copies/µl for each target species (Fig 1.1). The limit of detection (LoD) was determined to be $1x10^2$ copies/µl since concentrations below this value did not reproducibly produce a detectable signal. Similar efficiencies and LoDs were found for singleplex conditions compared to multiplex conditions.

Detection from artificially inoculated wood. Within PSHB associated fungi, *F. euwallaceae* was detected by the qPCR assay in 80%-100% of individual and mixed

fungal treatments that were inoculated with this species while it was recovered 40%-80% of the time from conventional isolation methods depending on the inoculation combination (Table 1.5). *G. euwallaceae* had a higher detection rate from qPCR (30%-80%) depending on the inoculation combination which was higher than conventional isolation (0-20%) (Table 1.5). *P. pembeum* was detected in 90% of treatments from qPCR assays from non-mixed treatments and only 10% from a mixed inoculation of *G. euwallaceae* and *P. pembeum* while the other treatments with this fungus were not detected by qPCR or conventional isolation (Table 1.5). For fungi associated with KSHB, *F. kuroshium* was detected by the qPCR assay from 70%-100% of individual and mixed fungal inoculations and 70% from conventional isolation. Detection of *G. kuroshium* from qPCR was 20-50% for individual and mixed inoculations compared to (0%-10%) from conventional methods (Table 1.5). Inoculations simulating a mix of fungi from both PSHB and KSHB resulted in 100% detection of *F. euwallaceae* and *F. kuroshium* from the respective qPCR assays and 70% of *Fusarium* spp. (undistinguished) from conventional isolation (Table 1.5). *G. euwallaceae* and *G. kuroshium* both resulted in detection of 80% from the qPCR assays while not being recovered from conventional isolation (Table 1.5).

User defined contrasts resulted in significantly greater starting quantities (SQ) ($P < 0.05$) of *G. euwallaceae* (TEX 615) from a mixed inoculation of *F. euwallaceae* (FAM) and *G. euwallaceae* compared to *G. euwallaceae* inoculated alone. *F. kuroshium* (HEX) SQ was significantly lower ($P < 0.05$) from a mixed inoculation of *F. kuroshium* and *G. kuroshium* (CY5.5™) compared to *F. kuroshium* inoculated alone. From mixed

inoculations of fungi from both PSHB and KSHB, *F. euwallaceae* was found to have significantly higher SQ ($P < 0.05$) in a mixed treatment of *F. euwallaceae* and *F. kuroshium* compared to *F. euwallaceae* inoculated alone. *Fusarium kuroshium* showed no significant differences in SQ ($P > 0.05$) in the same circumstance. *G. euwallaceae* was found to have significantly greater SQ ($P < 0.05$) in a mixed treatment of *G. euwallaceae* and *G. kuroshium* compared to *G. euwallaceae* inoculated alone while *G. kuroshium* showed no significant differences in SQ ($P > 0.05$) in the same circumstance (Table 1.6).

Quantification from beetle mycangia. Only *F. euwallaceae* and *G. euwallaceae* from PSHB heads and only *F. kuroshium* and *G. kuroshium* from KSHB heads (Fig. 1.2) were positively detected with the two qPCR assays. The SQ of *F. euwallaceae* in PSHB heads was found to be significantly greater ($P < 0.05$) than that of *G. euwallaceae*. In KSHB heads, the SQ of *F. kuroshium* was not found to be significantly greater ($P > 0.05$) than that of *G. kuroshium*. However, *F. euwallaceae* from PSHB had a significantly greater SQ than *F. kuroshium* in KSHB.

Recovery from field samples. Samples recovered from 65.4% of the locations sampled were positive for signs and symptoms of FD and confirmed by detecting fungi associated with the pests though conventional isolation methods. DNA extracted from wood samples failed to produce detectable signals for both assays and failed the SPUD test (Nolan et al. 2006) when positive controls were spiked with DNA extract from field samples. Due to the lack of PCR amplification after field samples were extracted for DNA, conventional isolation from infected wood was performed with downstream identification of recovered fungi by qPCR. Recovery rates from the three different plant species sampled, *P.*

racemosa, Populus spp., and *Salix* spp., were 88.3%, 72.7%, and 56.1% respectively for *Fusarium* spp. and 11.5%, 0%, and 21.1% respectively for *Graphium* spp. Samples positive for target *Fusarium* spp. were recovered significantly more ($P < 0.001$) than *Graphium* spp. from wood in all three plant species sampled (Fig. 1.3). Sample positive for *Fusarium* spp. was recovered significantly less ($P < 0.001$) in *Salix* spp. compared to *P. racemosa* sampled while *Graphium* spp. were recovered more in *Salix* spp. ($P = 0.075$) compared to *P. racemosa* (Fig. 1.3). Fungal isolates of *Fusarium* spp. and *Graphium* spp. from the both beetles were morphologically indistinguishable, therefore downstream identification of the growing fungi were identified using both PSHB and KSHB qPCR assays to detect all fungi associated with both beetles. Of the surveyed counties (Fig. 1.4), Orange county had both complexes present, in close proximity, mainly in the cities Lake Forest and Mission Viejo. On five trees in a four-mile radius within this region, both fungi associated with PSHB and KSHB were confirmed from the same tree. Both PSHB and KSHB were confirmed to be present in both Orange and San Diego Counties, and areas sampled in West Riverside County were all negative despite some trees exhibiting an entry hole, although PSHB has been found in northern parts of Riverside County in proximity to the Santa Ana River previous to this study (A. Eskalen, *unpublished data*).

DISCUSSION

Identification of PSHB and KSHB attacks can be difficult because the beetles are often absent from areas of visible attack and commonly mistaken for other benign ambrosia or bark beetles. In our current study, a real-time PCR assay was developed to address this issue and provide a rapid and reliable identification method to target specific

fungi associated with PSHB and KSHB, which is not dependent on the beetle being present in samples. Polymorphic regions of the β- tubulin locus (*TUB2)* were exploited to design, optimize, and validate two novel multiplexed qPCR assays which can qualitatively detect and quantitate all fungi associated with PSHB and KSHB respectively (Fig. 1.1, Fig. 1.5). The assays were shown to be sensitive with a LoD of $1x10^2$ copies/µl for all targets (Table 1.4) and able to detect targets across various sample types with *Fusarium* spp. shown to be the most frequent genera detected (Fig. 1.2,1.3; Table 1.5,1.6). However, DNA extraction from field samples of *P. racemosa, Populus* spp., and *Salix* spp. failed the SPUD test (Nolan et al. 2006), most likely due PCR inhibitors in the wood, therefore it is best to identify SHB attacks by isolating fungi from the wood and then using spores from recovered fungi as a template for the multiplexed qPCR assays. These real-time molecular tools are useful to diagnose and distinguish PSHB and KSHB from affected hosts in California to elucidate the distribution and movement of these pests as shown from our field surveys (Fig. 1.4).

For this study, we chose to exploit *TUB2* for our assays since it occurs as a single-copy gene in the genome of most fungi, and because this number is not likely to vary between isolated strains of a species, in contrast to gene regions like ITS (Lofgren et al. 2018; Longo et al. 2013; Kiss, 2012). While selection of a region with one copy number per genome can reduce the assay sensitivity, it also reduces quantification bias (Brancart et al. 2005) and allows conversion of copy numbers to individual spores. This makes the assay of high utility in diagnostic assays as spores are of abundance in female SHB mycangia (Freeman et al. 2016) and in fungal galleries from infested wood samples

(Lynch et al. 2016). The primers and hydrolysis probes selected were validated by AGE based on expected amplicon size along with specificity testing against *F. solani* and *F. oxysporum* isolates (Table 1.1), since these are two commonly recovered relative species recovered from SHB field samples coming from *P. americana, P. racemosa,* and *Salix* spp. (A. Eskalen, *unpublished data*). Primers and probes were also tested against other fungal species commonly recovered from xylem tissue of these hosts including *Alternaria alternata, Clonostachys rosea, Neofusicoccum parvum,* and *Penecillium digitatum* and confirmed non-specific amplification was not occurring from other fungi that may be present in SHB samples.

Multiplexed assays have been previously reported to be effective in detecting multiple plant pathogens from wood samples using real time methods (Li et al. 2006; Li, et al., 2009; Pouzoulet et al. 2013; Zitnick-Anderson, Simons, and Pasche, 2018), as well as detecting AFC fusaria associated with *Euwallacea* spp. from DNA extracts of fungi and beetle heads using conventional PCR methods (Short et al. 2017). The difference from the latter, is the assays presented here detect *all* known fungal symbionts associated with PSHB and KSHB in California from a sample, not solely targeting the AFC fusaria in these systems. In addition, detection of fungi from single beetle heads was successful with these assays, where pooled samples were the only sample types to show detection from the previously mentioned conventional multiplex PCR methods targeting AFC fusaria. Although AFC fusaria were recovered most frequently from various SHB samples in previous studies (Freeman et al. 2013; Freeman et al. 2016; Lynch et al. 2016; Mendel et al. 2012; Na et al. 2018; O'Donnell et al. 2015) as well as in this study,

detecting other fungi involved in these invasive pest disease complexes can increase identification success in samples from hosts such as *Salix* spp. which were shown to have greater abundance of *Graphium* spp. compared to other hosts tested. In previous studies, *P. pembeum* colony forming units (CFU) were found to be more abundant than *F. euwallaceae* in gallery samples from wood in *Acer negundo* and *Ricinus communis* while, overall, *G. euwallaceae* and *P. pembeum* were more abundant in galleries compared to beetle heads (Lynch et al. 2016). Due to the possibility of a higher abundance from fungal symbionts other than AFC fusaria, such as *Graphium* spp. and *P. pembeum,* in SHB samples, a system to detect *all* fungi associated with *Euwallacea* spp. would be more reliable for identification of these pest disease complexes in invaded areas like California.

Quantification of pathogen loads from DNA using multiplex assays has been done for plant pathogens from non-lignified tissue (Li, et al., 2009) as well as from lignified tissue (Pouzoulet et al. 2013), but to our knowledge, has not been applied to a SHB-FD system. The dynamics and function of the fungi associated with *Euwallacea* spp. has been discussed for AFC fusaria (Eskalen et al. 2013; Freeman et al. 2013; Freeman et al. 2016; Kasson et al. 2013; Lynch et al. 2016; Mendel et al. 2012; Na et al. 2018; O'Donnell et al. 2015) but less so for the function of *Graphium* spp. and *P. pembeum* in the system (Freeman et al. 2016; Lynch et al. 2016). Detection of all SHB-FD fungi from the multiplex assays were shown in DNA extracts from artificially inoculated avocado shoots and female beetle heads which had varying recovery rates (Fig 1.2; Table 1.5,1.6) but presents a platform to detect all fungi associated with PSHB and KSHB samples. The

PSHB and KSHB assays can be potentially utilized to compare relative quantity of SHB fungi within amplifiable samples to investigate effects of pesticide candidates on the quantity of these fungi from controlled *in vitro* and field experiments, possibly elucidating pesticides that disrupt the system for management of this invasive pest in California

Based on field samples collected from Orange, Riverside, and San Diego counties (Fig. 1.4), after identification using the multiplex assays, both PSHB and KSHB were present in northern San Diego county (Carlsbad) as well as the central-east side of Orange county (Irvine/Lake Forest), where five trees were found with fungi from both beetles within these areas. In previous studies it has been reported that ambrosia beetles can switch or steal fungal symbionts, known as promiscuous symbiosis or fungal sharing (Carrillo et al. 2014; Hulcr and Cognato, 2010; Kostovcik et al. 2015) and it is currently unknown if this phenomenon is occurring in areas such as Orange County which have trees that are supporting both PSHB and KSHB. Some invasive *Euwallacea* spp. have been reported to vector multiple AFC fusaria in Florida and Sri Lanka (O'Donnell et al. 2015). This raises concern for the situation in Orange County if PSHB and KSHB can accumulate new symbionts from each other, not limited to AFC fusaria, and potentially increase their arsenal to colonize woody hosts. Assessing which fungi are causing the damage becomes difficult since PSHB fungi were found to have significant differences in pathogenicity between fungi and host (Lynch et al. 2016; Na et al. 2018). Molecular tools such as the assays presented here have potential in assessing the dynamics of these fungi among the wide range of reproductive hosts, but the PCR inhibition in wood must be

overcome first. This may be due to hosts attacked in the field exhibiting varying conditions (age of gallery, host responses, extreme tissue discoloration) which may affect the samples and contribute to PCR inhibition that has been reported in other systems (Demke and Phan, 2009; Schaad and Frederick, 2002). However, the diagnostic assay can still be useful by using conventional isolation from suspect SHB samples and downstream qPCR identification of recovered fungi across a range of hosts (Fig. 1.2,1.3,1.4; Table 1.5,1.6) to detect the pest in early stages of infestation and aid in management decisions while reducing false identifications.

The invasion of *Euwallacea* spp. from Southeast Asia has occurred in many places around the world (CABI 2015; Eskalen et al. 2013; Hulcr and Stelinski et al. 2017, O'Donnell et al. 2015; Paap et al. 2018; Short et al. 2017) with humans potentially having a significant impact on moving this pest around unintentionally from infested areas into un-infested areas. This can happen through movement of infested nursery materials and firewood that may contain live beetles (Sanborn 1996; Haack, Petrice and Weidenhoeft 2010). For instance, KSHB was positively identified in Santa Barbara from suspect *P. racemosa* wood using these assays (Data not shown), which was unexpected since KSHB had previously not been detected northern part of Orange County nor anywhere in Los Angeles county. The beetles were also trapped and identified (Rugman-Jones and Stouthamer, 2017) in this area as well as San Luis Obispo. It is still unknown how KSHB spread in these areas, undetected throughout Los Angeles county since KSHB has concentrated populations south of Los Angeles County. Diagnosing these suspect samples and understanding the distribution of these pests provides insight into their

spread (Fig. 1.4) and provides useful information to inform the land managers and public to help prevent further movement of infested material. Some potential pesticides treatment options have been researched including injection and spray applications with insecticides, fungicides, and a biopesticide on sycamore in California (Mayorquin et al. 2018) with some found to be effective in reducing further infestation of the beetles compared to the controls, providing arborists, nurseries, and land managers with options to prevent further infestation. Early detection can increase the survival or affected hosts while reducing the spread of these beetles into new areas by reducing their accumulation on hosts. This quantitative method provides a baseline platform to identify PSHB and KSHB fungi in California, but it would be interesting to see a qPCR approach applied to other *Euwallacea* spp. systems in other invaded areas. Areas such as Israel and South Africa are reported to have PSHB (Mendel et al. 2012; Paap et al. 2018), therefore the PSHB triplex assay may be utilized in these areas to diagnose samples. Future studies should consider both identifying the beetles and fungi in a given SHB-FD complex in one sample if the hydrolysis probe combinations will permit it. Quantitative approaches can aid in understanding dynamics of SHB fungal mutualist and functions in their life cycle as well as our ability to diagnose plants and manage infestations through early detection in order to prevent spread of these invasive pests on a regional and global scale.

LITERATURE CITED

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol., 215(3), 403-410.

Atallah, Z.K., Bae, J., Jansky, S.H., Rouse, D.I. and Stevenson, W.R., 2007. Multiplex real-time quantitative PCR to detect and quantify *Verticillium dahliae* colonization in potato lines that differ in response to Verticillium wilt. Phytopathology, 97(7), 865-872.

Beaver, R.A., Sittichaya, W. and Liu, L.Y., 2014. A synopsis of the scolytine ambrosia beetles of Thailand (Coleoptera: Curculionidae: Scolytinae). Zootaxa, 3875(1), 1-82.

Biassoni, R. and Raso, A., 2014. Quantitative Real-Time PCR. Humana Press.

Billones-Baaijens, R., Úrbez-Torres, J.R., Liu, M., Ayres, M., Sosnowski, M. and Savocchia, S., 2018. Molecular Methods to Detect and Quantify Botryosphaeriaceae Inocula Associated With Grapevine Dieback in Australia. Plant Dis., 1489-1499.

Bodles, W.J., Fossdal, C.G. and Woodward, S., 2006. Multiplex real-time PCR detection of pathogen colonization in the bark and wood of *Picea sitchensis* clones differing in resistance to Heterobasidion annosum. Tree Physiol., 26(6), 775-782.

Boland, J.M., 2016. The impact of an invasive ambrosia beetle on the riparian habitats of the Tijuana River Valley, California. PeerJ, 4, e2141.

Brancart, F., Rodriguez-Villalobos, H., Fonteyne, P.A., Peres-Bota, D. and Liesnard, C., 2005. Quantitative TaqMan PCR for detection of Pneumocystis jiroveci. Journal of microbiological methods, 61(3), 381-387.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L. and Vandesompele, J., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry, 55(4), 611-622.

CABI 2015. *Euwallacea fornicatus* Distribution Map. http://www.cabi.org/isc/datasheet/57163.

Carrillo, D., Duncan, R.E., Ploetz, J.N., Campbell, A.F., Ploetz, R.C. and Peña, J.E., 2014. Lateral transfer of a phytopathogenic symbiont among native and exotic ambrosia beetles. Plant Pathol., 63(1), 54-62.

Carrillo, D., Narvaez, T., Cossé, A.A., Stouthamer, R. and Cooperband, M., 2015. Attraction of *Euwallacea* nr. *fornicatus* (Coleoptera: Curculionidae: Scolytinae) to lures containing quercivorol. Fla. Entomol., 98(2), 780-782.

Chamberlain, J.S., Gibbs, R.A., Rainer, J.E., Nguyen, P.N. and Thomas, C., 1988. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res., 16(23), 11141-11156.

Chandelier, A., Massot, M., Fabreguettes, O., Gischer, F., Teng, F. and Robin, C., 2018. Early detection of Cryphonectria parasitica by real-time PCR. Eur. J. Plant Pathol., 1-18.

Cheng, J., Karambelkar, B., Xie, Y., Wickham, H., Russell, K. and Johnson, K., 2018. Leaflet: create interactive web maps with the JavaScript "Leaflet" Library. 2017.

Cooperband, M.F., Stouthamer, R., Carrillo, D., Eskalen, A., Thibault, T., Cossé, A.A., Castrillo, L.A., Vandenberg, J.D. and Rugman-Jones, P.F., 2016. Biology of two members of the *E uwallacea fornicatus* species complex (C oleoptera: C urculionidae: S colytinae), recently invasive in the USA, reared on an ambrosia beetle artificial diet. Agr. Forest Entomol., 18(3), 223-237.

Demeke, T., Ratnayaka, I. and Phan, A., 2009. Effects of DNA extraction and purification methods on real-time quantitative PCR analysis of Roundup Ready soybean. Journal of AOAC International, 92(4), 1136-1144.

Eskalen, A., Stouthamer, R., Lynch, S. C., Rugman-Jones, P. F., Twizeyimana, M., Gonzalez, A., & Thibault, T. 2013. Host Range of Fusarium Dieback and Its Ambrosia Beetle (Coleoptera: Scolytinae) Vector in Southern California. Plant Dis., 97(7), 938–951.

Freeman, S., Sharon, M., Maymon, M., Mendel, Z., Protasov, A., Aoki, T., Eskalen, A., O'Donnell, K. 2013. *Fusarium euwallaceae* sp. nov.--a symbiotic fungus of *Euwallacea sp*., an invasive ambrosia beetle in Israel and California. Mycologia, 105(6), 1595–1606.

Freeman, S., Sharon, M., Dori-Bachash, M., Maymon, M., Belausov, E., Maoz, Y., Margalit, O., Protasov, A. and Mendel, Z., 2016. Symbiotic association of three fungal species throughout the life cycle of the ambrosia beetle *Euwallacea* nr. *fornicatus*. Symbiosis, 68(1-3), 115-128.

Glass, N.L. and Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microb., 61(4), 1323-1330.

Haack, R.A., Petrice, T.R. and Wiedenhoeft, A.C., 2010. Incidence of bark-and wood-boring insects in firewood: a survey at Michigan's Mackinac Bridge. Journal of Economic Entomology, 103(5), 1682-1692.

Heid, C.A., Stevens, J., Livak, K.J. and Williams, P.M., 1996. Real time quantitative PCR. Genome Res., 6(10), 986-994.

Heller, W.P. and Keith, L.M., 2018. Real-Time PCR Assays to Detect and Distinguish the Rapid 'Ōhi'a Death Pathogens Ceratocystis lukuohia and C. huliohia. Phytopathology, 108(12), 1395-1401.

Henegariu, O., Heerema, N.A., Dlouhy, S.R., Vance, G.H. and Vogt, P.H., 1997. Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques, 23(3), 504-511.

Higuchi, R., Dollinger, G., Walsh, P.S. and Griffith, R., 1992. Simultaneous amplification and detection of specific DNA sequences. Nat. Biotechnol., 10(4), 413.

Hofstetter, R. W., Dinkins-Bookwalter, J., Davis, T. S., & Klepzig, K. D. 2015. Symbiotic Associations of Bark Beetles. Bark Beetles: Biology and Ecology of Native and Invasive Species. Elsevier Inc.

Hothorn, T., Bretz, F. and Westfall, P., 2008. Simultaneous inference in general parametric models. Biometrical J., 50(3), 346-363.

Hulcr, J., & Cognato, A. I. 2010. Repeated evolution of crop theft in fungus-farming ambrosia beetles. Evolution, 64(11), 3205–3212.

Hulcr, J., & Stelinski, L. L. 2017. The Ambrosia Symbiosis: From Evolutionary Ecology to Practical Management. Annu. Rev. Entomol., (0).

Kasson, M. T., O'Donnell, K., Rooney, A. P., Sink, S., Ploetz, R. C., Ploetz, J. N., Konkol, J. L., Carrillo, D., Freeman, S., Mendel, Z., Smith, J. A., Black, A. W., Hulcr, J., Bateman, C., Stefkova, K., Campbell, P. R., Geering, A. D. W., Dann, E. K., Eskalen, A., Mohotti, K., Short, D. P. G., Aoki, T., Fenstermacher, K. A., Davis, D. D., Geiser, D. M. 2013. An inordinate fondness for *Fusarium*: Phylogenetic diversity of fusaria cultivated by ambrosia beetles in the genus *Euwallacea* on avocado and other plant hosts. Fungal Genet. Biol., 56, 147–157.

Kiss, L., 2012. Limits of nuclear ribosomal DNA internal transcribed spacer (ITS) sequences as species barcodes for Fungi. P. Natl. Acad. Sci, 109(27), E1811-E1811.

Kleiber, C., Zeileis, A. and Zeileis, M.A., 2018. Package 'AER'. *R package version 1.2*, 4.

Koressaar, T. and Remm, M., 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics, 23(10), 1289-1291.

Kostovcik, M., Bateman, C.C., Kolarik, M., Stelinski, L.L., Jordal, B.H. and Hulcr, J., 2015. The ambrosia symbiosis is specific in some species and promiscuous in others: evidence from community pyrosequencing. ISME J, 9(1), 126.

Li, W., Hartung, J. S., & Levy, L. (2006). Quantitative real-time PCR for detection and identification of *Candidatus Liberibacter* species associated with citrus huanglongbing. J. Microbiol. Meth., 66, 104–115.

Li, W., Abad, J.A., French-Monar, R.D., Rascoe, J., Wen, A., Gudmestad, N.C., Secor, G.A., Lee, M., Duan, Y. and Levy, L., 2009. Multiplex real-time PCR for detection, identification and quantification of '*Candidatus Liberibacter solanacearum*'in potato plants with zebra chip. J. Microbiol. Meth., 78(1), 59-65.

Lofgren, L.A., Uehling, J.K., Branco, S., Bruns, T.D., Martin, F. and Kennedy, P.G., 2018. Genome-based estimates of fungal rDNA copy number variation across phylogenetic scales and ecological lifestyles. Molecular ecology.

Longo, A.V., Rodriguez, D., da Silva Leite, D., Toledo, L.F., Almeralla, C.M., Burrowes, P.A. and Zamudio, K.R., 2013. ITS1 copy number varies among *Batrachochytrium dendrobatidis* strains: implications for qPCR estimates of infection intensity from field-collected amphibian skin swabs. PLoS One, 8(3), e59499.

Lynch, S. C., Twizeyimana, M., Mayorquin, J. S., Wang, D. H., Na, F., Kayim, M., ... & Hulcr, J. 2016. Identification, pathogenicity and abundance of *Paracremonium pembeum* sp. nov. and *Graphium euwallaceae* sp. nov.—two newly discovered mycangial associates of the polyphagous shot hole borer (*Euwallacea sp*.) in California. Mycologia, 108(2), 313-329.

Malvick, D.K. and Grunden, E., 2005. Isolation of fungal DNA from plant tissues and removal of DNA amplification inhibitors. Mol. Ecol. Notes, 5(4), 958-960.

Mayorquin, J.S., Carrillo, J.D., Twizeyimana, M., Peacock, B.B., Sugino, K.Y., Na, F., Wang, D.H., Kabashima, J.N. and Eskalen, A., 2018. Chemical Management of Invasive Shot Hole Borer and Fusarium Dieback in California Sycamore (*Platanus racemosa*) in Southern California. Plant Dis., 102(7), 1307-1315.

Mendel, Z., Protasov, A., Sharon, M., Zveibil, A., Yehuda, S.B., O'Donnell, K., Rabaglia, R., Wysoki, M. and Freeman, S., 2012. An Asian ambrosia beetle *Euwallacea fornicatus* and its novel symbiotic fungus *Fusarium* sp. pose a serious threat to the Israeli avocado industry. Phytoparasitica, 40(3), 235-238.

Na, F., Carrillo, J.D., Mayorquin, J.S., Ndinga-Muniania, C., Stajich, J.E., Stouthamer, R., Huang, Y.T., Lin, Y.T., Chen, C.Y. and Eskalen, A., 2018. Two novel fungal symbionts *Fusarium kuroshium* sp. nov. and *Graphium kuroshium* sp. nov. of Kuroshio shot hole borer (*Euwallacea* sp. nr. *fornicatus*) cause Fusarium dieback on woody host species in California. Plant Dis., 102(6),1154-1164.

Nolan, T., Hands, R.E., Ogunkolade, W. and Bustin, S.A., 2006. SPUD: a quantitative PCR assay for the detection of inhibitors in nucleic acid preparations. Anal. Biochem., 351(2), 308-310.

OC Parks 2016. Shot Hole Borer: Managing the Invasive Beetle. https://oc-parks-gis.maps.arcgis.com/apps/Cascade/index.html?appid=680fd0c9e73f4857a8477791f7ee796f.

O'Donnell, K., Sink, S., Libeskind-Hadas, R., Hulcr, J., Kasson, M. T., Ploetz, R. C., Konkol, J. L., Ploetz, J. N., Carrillo, D., Campbell, A., Duncan, R. E., Liyanage, P. N., Eskalen, A., Na, F., Geiser, D. M., Bateman, C., Freeman, S., Mendel, Z., Sharon, M., Aoki, T., Crossé, A. A., Rooney, A. P. 2015. Discordant phylogenies suggest repeated host shifts in the *Fusarium-Euwallacea* ambrosia beetle mutualism. Fungal Genet. Biol., 82, 277–290.

O'Donnell, K., Libeskind-Hadas, R., Hulcr, J., Bateman, C., Kasson, M.T., Ploetz, R.C., Konkol, J.L., Ploetz, J.N., Carrillo, D., Campbell, A. and Duncan, R.E., 2016. Invasive Asian *Fusarium–Euwallacea* ambrosia beetle mutualists pose a serious threat to forests, urban landscapes and the avocado industry. Phytoparasitica, 44(4), 435-442.

Oliveira, A.C., Vallim, M.A., Semighini, C.P., Araújo, W.L., Goldman, G.H. and Machado, M.A., 2002. Quantification of *Xylella fastidiosa* from citrus trees by real-time polymerase chain reaction assay. Phytopathology, 92(10), 1048-1054.

Osman, F. and Rowhani, A., 2006. Application of a spotting sample preparation technique for the detection of pathogens in woody plants by RT-PCR and real-time PCR (TaqMan). J. Virol. Methods, 133(2), 130-136.

Paap, T., De Beer, Z.W., Migliorini, D., Nel, W.J. and Wingfield, M.J., 2018. The polyphagous shot hole borer (PSHB) and its fungal symbiont *Fusarium euwallaceae*: a new invasion in South Africa. Australas. Plant Path., 47(2), 231-237.

Palacio-Bielsa, A., Cambra, M.A. and López, M.M., 2009. PCR detection and identification of plant-pathogenic bacteria: updated review of protocols (1989-2007). J. Plant Pathol., 249-297.

Pearson, W.R., 2016. Finding protein and nucleotide similarities with FASTA. Current protocols in bioinformatics, 53(1), 3-9.

Postollec, F., Falentin, H., Pavan, S., Combrisson, J. and Sohier, D., 2011. Recent advances in quantitative PCR (qPCR) applications in food microbiology. Food Microbiol., 28(5), 848-861.

Pouzoulet, J., Mailhac, N., Couderc, C., Besson, X., Daydé, J., Lummerzheim, M. and Jacques, A., 2013. A method to detect and quantify *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* DNA in grapevine-wood samples. Appl. Microbiol. Biot., 97(23), 10163-10175.

Qu, X.S., Wanner, L.A. and Christ, B.J., 2011. Multiplex real-time PCR (TaqMan) assay for the simultaneous detection and discrimination of potato powdery and common scab diseases and pathogens. J. Appl. Microbiol., 110(3), 769-777.

Rugman-Jones, P.F. and Stouthamer, R., 2017. High-resolution melt analysis without DNA extraction affords rapid genotype resolution and species identification. Mol. Ecol. Resour., 17(4), 598-607.

Sanborn, S.R., 1996. *Controlling bark beetles in wood residue and firewood*. California Department of Forestry and Fire Protection.

Schaad, N.W. and Frederick, R.D., 2002. Real-time PCR and its application for rapid plant disease diagnostics. Canadian journal of plant pathology, 24(3), 250-258.

Schena, L., Nigro, F., Ippolito, A. and Gallitelli, D., 2004. Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. Eur. J. Plant Pathol., 110(9), 893-908.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., Bolchacova, E., Voigt, K., Crous, P.W. and Miller, A.N., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. P. Natl. Acad. Sci, 109(16), 6241-6246.

Schrader, C., Schielke, A., Ellerbroek, L. and Johne, R., 2012. PCR inhibitors–occurrence, properties and removal. J. Appl. Microbiol., 113(5), 1014-1026.

Short, D.P., O'Donnell, K., Stajich, J.E., Hulcr, J., Kijimoto, T., Berger, M.C., Macias, A.M., Spahr, E.J., Bateman, C.C., Eskalen, A. and Lynch, S.C., 2017. PCR Multiplexes Discriminate *Fusarium* Symbionts of Invasive *Euwallacea* Ambrosia Beetles that Inflict Damage on Numerous Tree Species Throughout the United States. Plant Dis., 101(1), 233-240.

Stouthamer, R., Rugman-Jones, P., Thu, P.Q., Eskalen, A., Thibault, T., Hulcr, J., Wang, L.J., Jordal, B.H., Chen, C.Y., Cooperband, M. and Lin, C.S., 2017. Tracing the origin of a cryptic invader: phylogeography of the E uwallacea fornicatus (C oleoptera: C urculionidae: S colytinae) species complex. Agricultural and forest entomology, 19(4), 366-375.

Team, R.C., 2013. R: A language and environment for statistical computing. https://www.gbif.org/tool/81287/r-a-language-and-environment-for-statistical-computing

Tellenbach, C., Grünig, C.R. and Sieber, T.N., 2010. Suitability of quantitative real-time PCR to estimate the biomass of fungal root endophytes. Appl. Environ. Microb., 76(17), 5764-5772.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. 1997. The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res., 25(24), 4876–4882.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M. and Rozen, S.G., 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res., 40(15), e115-e115.

Venables, W.N. and Ripley, B.D., 2002. Tree-based methods. Modern Applied Statistics with S (251-269). Springer, New York, NY.

Wickham, H., 2011. The split-apply-combine strategy for data analysis. J. Stat. Softw., 40(1), 1-29.

Wickham, H., 2016. ggplot2*:* elegant graphics for data analysis. Springer.

White, T.J., Bruns, T., Lee, S.J.W.T. and Taylor, J.L., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 18(1), 315-322.

Zitnick-Anderson, K., Simons, K. and Pasche, J.S., 2018. Detection and qPCR quantification of seven *Fusarium* species associated with the root rot complex in field pea. Can. J. Plant Pathol., 40(2), 261-271.

Table 1.1. Representative fungi from *E. whitfordiodendrus* and *E. kuroshio* and various hosts in California used in this study

					GenBank numbers[a]	
Isolate	**Species**	**Host**	**Location[b]**	**SHB Complex**	**β-tubulin**	**ITS**
NRRL62626[ab]	*Fusarium euwallaceae*	*Persea americana*	Los Angeles, CA	PSHB	KU171782	…
UCR4511	*F. euwallaceae*	*Platanus racemosa*	Riverside, CA	PSHB	MK108959	…
UCR4920[a]	*F. euwallaceae*	*Eucalyptus sp.*	Lake Forest, CA	PSHB	MK108960	…
UCR4937	*F. euwallaceae*	*Salix lasiolepis*	Lake Forest, CA	PSHB	MK108961	…
NRRL54723[b]	*F. euwallaceae*	*P. americana*	Glil Yam, Israel	PSHB	MK108962	…
UCR2974	*Graphium euwallaceae*	*Ricinus communis*	Los Angeles, CA	PSHB	MK108968	…
UCR2977	*G. euwallaceae*	*Acacia floribunda*	Los Angeles, CA	PSHB	MK108969	…
UCR2979	*G. euwallaceae*	*Quercus agrifolia*	Los Angeles, CA	PSHB	MK108970	…
UCR2980[a]	*G. euwallaceae*	*P. americana*	Los Angeles, CA	PSHB	MK108971	…
UCR2981[a]	*G. euwallaceae*	*P. americana*	Los Angeles, CA	PSHB	MK108972	…
UCR2983[a]	*Paracremonium pembeum*	*P. americana*	Los Angeles, CA	PSHB	KU053056	…
UCR2989	*P. pembeum*	*R. communis*	Los Angeles, CA	PSHB	KU053062	…
UCR2990	*P. pembeum*	*R. communis*	Los Angeles, CA	PSHB	KU053063	…
UCR2992	*P. pembeum*	*P. americana*	Los Angeles, CA	PSHB	KU053065	…
UCR2993[a]	*P. pembeum*	*R. communis*	Los Angeles, CA	PSHB	KU053066	…
CBS142642[ac]	*F. kuroshium*	*Platanus racemosa*	El Cajon, CA	KSHB	MK108963	…
UCR3644[a]	*F. kuroshium*	*P. racemosa*	El Cajon, CA	KSHB	MK108964	…
UCR3651	*F. kuroshium*	*E. kuroshio*	Fallbrook, CA	KSHB	MK108965	…
UCR3653	*F. kuroshium*	*P. americana*	Bonsall, CA	KSHB	MK108966	…
UCR3654	*F. kuroshium*	*P. americana*	Bonsall, CA	KSHB	MK108967	…
CBS142643[ac]	*G. kuroshium*	*P. americana*	Fallbrook, CA	KSHB	MK108973	…
UCR4594[a]	*G. kuroshium*	*P. americana*	Fallbrook, CA	KSHB	MK108974	…
UCR4606	*G. kuroshium*	*P. americana*	Bonsall, CA	KSHB	MK108975	…
UCR4607	*G. kuroshium*	*P. americana*	Bonsall, CA	KSHB	MK108976	…
UCR4608	*G. kuroshium*	*Euwallacea kuroshio*	Bonsall, CA	KSHB	MK108977	…
UCR7265	*F. solani*	*P. americana*	Escondido, CA	N/A	…	MK101041
UCR7266	*F. solani*	*P. racemosa*	Irvine, CA	N/A	…	MK101040
NRRL13414[b]	*F. solani*	N/A	California, USA	N/A	…	…
NRRL22253[b]	*F. solani*	*Prunus persica*	Hebei, China	N/A	…	…
NRRL22639[b]	*F. solani*	*Allium cepa*	Canada	N/A	…	…
UCR3424	*F. oxysporum*	*P. americana*	Irvine, CA	N/A	…	MK101043
UCR6846	*F. oxysporum*	*P. americana*	Riverside, CA	N/A	…	MK101042
NRRL38352[b]	*F. oxysporum*	*Solanum tuberosum*	Texas, USA	N/A	…	…
NRRL38357[b]	*F. oxysporum*	*Eustoma sp.*	Florida, USA	N/A	…	…
NRRL34118[b]	*F. oxysporum* f. sp. *vasinfectum*	N/A	California, USA	N/A	…	…
NRRL22678[b]	*F. brasiliense*	*Phaseolus vulgaris*	California, USA	N/A	…	FJ919501
UCR2575	*Colletotrichum gloeosporoides*	*Citrus reticulata*	California, USA	N/A	…	MK447629
UCR7052	*Alternaria alternata*	*P. americana*	California, USA	N/A	…	MK101044
UCR5314	*Neofusicoccum parvum*	*Salix lasiolepis*	California, USA	N/A	…	MH191115
UCR3250	*Clonostachys rosea*	*P. americana*	California, USA	N/A	…	MK101045
UCR7070	*Penicillium expansum*	*P. americana*	California, USA	N/A	…	MK101046

[a] Isolates used in wood inoculation

[b] Isolates from Agricultural Research Service (ARS) culture collection, United States

[c] Isolates from CBS-KNAW Culture Collection, The Netherlands

Table 1.2. List of the oligonucleotides used in this study

Oligo ID	Sequence (5'→ 3') with modifications[a]	Product Length (bp)	T_m (°C)	GC (%)	Assay Type
FUEU-btF	GTTACCTGACCTGCTCTGC**C**	76	57.9	60	PSHB
FUEU-btR	ACGGCTGGGAAAT**G**TTAG**C**	…	55.6	52.6	PSHB
FUEU-bt-probe	/56-FAM/AGTTTTG**T**T/ZEN/**T**TGACATT**G**GT**C**GA**G**CAA/3IABkFQ/	…	59.9	37	PSHB
GREU-btF	G**A**TACCTCACCTGCTCTGCT	143	56.4	55	PSHB
GREU-btR	ACCACGGCTATTGTG**AA**C**A**G	…	55.2	50	PSHB
GREU-bt-probe	/5TEX615/TGCTGCTTG**C**TGTGCT**C**GA**T**TTGTTC/3IAbRQSp/	…	62.1	50	PSHB
PAPE-btF	CAGTGCGTAAGTAGCCGACTC	126	57.5	57.1	PSHB
PAPE-btR	GAGATGGTCTGCCAGAAAGC	…	55.9	55	PSHB
PAPE-bt-probe	/5Cy55/ATACTGACGATGCTTCACAGGGTAACC/3IAbRQSp/	…	60	48.2	PSHB
FUKU-btF	GTTACCTGACCTGCTCTGC**T**	78	56.8	55	KSHB
FUKU-btR	CCACGGCTGGGAAAT**A**TTAG**T**	…	54.7	47.6	KSHB
FUKU-bt-probe	/5HEX/AGTTTTG**G**T/ZEN/**C**TGACATT**T**GT**T**GA**A**CAA/3IABkFQ/	…	58.6	37	KSHB
GRKU-btF	G**C**TACCTCACCTGCTCTGCT	142	59.2	60	KSHB
GRKU-btR	CCACGGCTATTGTGAA**G**A**A**A**C**	…	54.1	47.6	KSHB
GRKU-bt-probe	/5Cy5/TGCTGCTTG**T**TGTGCT**T**GA**C**TTGTTC/3IAbRQSp/	…	60.4	46.2	KSHB

[a] Letters in bold indicate single nucleotide polymorphisms between the members of the same genus

Table 1.3. Comparison of Cq values from each target species in singleplex and multiplex reactions

Concentration (copies/µl)[a]	Assay Type	Reaction Type	Target Species	Florophore	Cq Values (µ±σ)
1000000	PSHB complex	Singleplex	*Fusarium euwallaceae*	FAM	18.56 ± 0.06
1000000	PSHB complex	Multiplex	*F.euwallaceae*	FAM	18.11 ± 0.02
1000000	PSHB complex	Singleplex	*Graphium euwallaceae*	TEX 615	17.56 ± 0.03
1000000	PSHB complex	Multiplex	*G. euwallaceae*	TEX 615	17.66 ± 0.27
1000000	PSHB complex	Singleplex	*Paracremonium pembeum*	Cy5.5™	18.09 ± 0.06
1000000	PSHB complex	Multiplex	*P. pembeum*	Cy5.5™	17.37 ± 0.11
1000000	KSHB complex	Singleplex	*F. kuroshium*	HEX	18.86 ± 0.46
1000000	KSHB complex	Multiplex	*F. kuroshium*	HEX	18.49 ± 0.13
1000000	KSHB complex	Singleplex	*G. kuroshium*	Cy5™	18.19 ± 0.36
1000000	KSHB complex	Multiplex	*G. kuroshium*	Cy5™	18.07 ± 0.15
100	PSHB complex	Singleplex	*F.euwallaceae*	FAM	31.06 ± 0.12
100	PSHB complex	Multiplex	*F.euwallaceae*	FAM	30.89 ± 0.12
100	PSHB complex	Singleplex	*G. euwallaceae*	TEX 615	30.64 ± 0.30
100	PSHB complex	Multiplex	*G. euwallaceae*	TEX 615	30.94 ± 0.41
100	PSHB complex	Singleplex	*P. pembeum*	Cy5.5™	30.77 ± 0.14
100	PSHB complex	Multiplex	*P. pembeum*	Cy5.5™	29.79 ± 0.33
100	KSHB complex	Singleplex	*F. kuroshium*	HEX	32.42 ± 0.30
100	KSHB complex	Multiplex	*F. kuroshium*	HEX	32.63 ± 0.29
100	KSHB complex	Singleplex	*G. kuroshium*	Cy5™	32.73 ± 0.19
100	KSHB complex	Multiplex	*G. kuroshium*	Cy5™	32.05 ± 0.54

[a] Concentrations derived from diluted gBlocks suspensions

Table 1.4. Evidence of reproducibility across experimental plates

Concentration (copies/µl)[a]	Assay Type	Target Species	Florophore	Cq Values (µ±σ)	CV(%)	Cq Values (µ±σ)	CV(%)
1000000	PSHB complex	*Fusarium euwallaceae*	FAM	18.00 ± 0.33	1.86	18.07 ± 0.30	1.66
100000	PSHB complex	*F.euwallaceae*	FAM	21.10 ± 0.27	1.36	21.19 ± 0.19	0.91
10000	PSHB complex	*F.euwallaceae*	FAM	24.44 ± 0.14	0.57	24.53 ± 0.20	0.82
1000	PSHB complex	*F.euwallaceae*	FAM	27.94 ± 0.14	0.51	27.98 ± 0.33	1.19
100	PSHB complex	*F.euwallaceae*	FAM	30.54 ± 0.03	0.09	30.75 ± 0.36	1.19
1000000	PSHB complex	*Graphium euwallaceae*	TEX 615	17.75 ± 0.29	1.66	17.41 ± 0.39	2.24
100000	PSHB complex	*G. euwallaceae*	TEX 615	20.57 ± 0.21	1.04	20.42 ± 0.32	1.57
10000	PSHB complex	*G. euwallaceae*	TEX 615	23.80 ± 0.34	1.43	23.73 ± 0.35	1.47
1000	PSHB complex	*G. euwallaceae*	TEX 615	27.20 ± 0.43	1.59	27.01 ± 0.45	1.65
100	PSHB complex	*G. euwallaceae*	TEX 615	30.05 ± 0.43	1.44	30.02 ± 0.37	1.23
1000000	PSHB complex	*Paracremonium pembeum*	Cy5.5™	17.40 ± 0.23	1.30	17.49 ± 0.29	1.68
100000	PSHB complex	*P. pembeum*	Cy5.5™	20.68 ± 0.20	0.98	20.68 ± 0.38	1.82
10000	PSHB complex	*P. pembeum*	Cy5.5™	24.17 ± 0.14	0.57	24.10 ± 0.30	1.25
1000	PSHB complex	*P. pembeum*	Cy5.5™	27.71 ± 0.54	1.95	27.55 ± 0.39	1.43
100	PSHB complex	*P. pembeum*	Cy5.5™	30.70 ± 0.37	1.23	30.76 ± 0.28	0.92
1000000	KSHB complex	*Fusarium kuroshium*	HEX	18.38 ± 0.15	0.83	18.41 ± 0.20	1.09
100000	KSHB complex	*F. kuroshium*	HEX	22.18 ± 0.21	1.93	21.9 ± 0.49	2.26
10000	KSHB complex	*F. kuroshium*	HEX	25.50 ± 0.32	1.27	25.39 ± 0.27	1.04
1000	KSHB complex	*F. kuroshium*	HEX	28.95 ± 0.69	2.38	29.19 ± 0.57	1.96
100	KSHB complex	*F. kuroshium*	HEX	32.18 ± 0.22	0.68	32.72 ± 0.50	1.54
1000000	KSHB complex	*Graphium kuroshium*	Cy5™	18.18 ± 0.17	0.95	18.14 ± 0.21	1.16
100000	KSHB complex	*G. kuroshium*	Cy5™	21.44 ± 0.35	0.35	21.36 ± 0.53	2.50
10000	KSHB complex	*G. kuroshium*	Cy5™	24.80 ± 0.14	0.55	24.7 ± 0.34	1.38
1000	KSHB complex	*G. kuroshium*	Cy5™	28.43 ± 0.57	1.99	28.48 ± 0.47	1.64
100	KSHB complex	*G. kuroshium*	Cy5™	31.63 ± 0.88	2.81	31.95 ± 0.94	2.95

[a] Concentrations derived from diluted gBlocks suspensions

[b] Intra-assay Cq values were generated across 1 experimental plate (N =3)

[c] Inter-assay Cq values were generated across 6 experimental plates (N =18)

Table 1.5. Comparison of recovery and quantification of fungi from detached shoots inoculated with SHB fungi

Treatment[a]	**SHB Complex**[b]	**% Recovery (Isolation)**[c]	**% Recovery (qPCR)**[d]
F. euwallaceae	PSHB	80	100
F. euwallaceae/ G. euwallaceae	PSHB	40/20	100/30
F. euwallaceae/ P. pembeum	PSHB	60/0	80/0
F. euwallaceae/ G. euwallaceae/ P. Pembeum	PSHB	50/0/0	100/20/0
G. euwallaceae	PSHB	0	60
G. euwallaceae/ P. pembeum	PSHB	10/10	80/0
P. pembeum	PSHB	0	90
F. kuroshium	KSHB	70	100
F. kuroshium/ G. kuroshium	KSHB	70/10	70/20
G. kuroshium	KSHB	0	50
F. euwallaceae/ F. kuroshium	N/A	70	100/100
G. euwallaceae/ G. kuroshium	N/A	0/0	80/80

[a] All inoculations were done on detached shoots (*N=10*) by depositing 1000 spores of each species into artificial wounds

[b] Mixed inoculations of species from both complexes have not been observed in nature, therfore are represented with "N/A"

[c] All isolations were done on PDA amended with 0.01% tetracycline hydrochloride

[d] Recovery values are from qualatative results

Table 1.6. Quantification of fungi from detached shoots inoculated with SHB fungi

			***P* Value**[d]				
Treatment[a]	**SHB Complex**[b]	**Estimated Starting Quantity (μ±σ)**[c]	**FAM**	**TEX 615**	**Cy5.5™**	**HEX**	**Cy5™**
F. euwallaceae	PSHB	596.8 ± 213.5	*Ref*	…	…	…	…
F. euwallaceae/ G. euwallaceae	PSHB	470.9 ± 304.47/ 352.4 ± 221.2	0.634	**0.002**	…	…	…
F. euwallaceae/ P. pembeum	PSHB	1242.6 ± 607/ N/A	0.172	…	N/A	…	…
F. euwallaceae/ G. euwallaceae/ P. pembeum	PSHB	876.2 ± 235/ 137.9 ± 17.2/ N/A	0.439	0.462	N/A	…	…
G. euwallaceae	PSHB	108.51 ± 12.3	…	*Ref*	…	…	…
G. euwallaceae/ P. pembeum	PSHB	131.4 ± 15.7/ N/A	…	0.387	N/A	…	…
P. pembeum	PSHB	119.1 ± 25.6	…	…	*Ref*	…	…
F. kuroshium	KSHB	2287.5 ± 534.2	…	…	…	*Ref*	…
F. kuroshium/ G. kuroshium	KSHB	1260.3 ± 667.9/ 152.4 ± 28.4	…	…	…	**<0.001**	0.887
G. kuroshium	KSHB	170.4 ± 55	…	…	…	…	*Ref*
F. euwallaceae/ F. kuroshium	N/A	1593.6 ± 594.5/ 3355 ± 1086.9	**0.048**	…		0.700	…
G. euwallaceae/ G. kuroshium	N/A	171 ± 93.3/ 344.5 ± 174.8	…	**0.012**	…	…	0.167

[a] All inoculations were done on detached shoots (*N=10*) by depositing 1000 spores of each species into artificial wounds

[b] Mixed inoculations of species from both complexes have not been observed in nature, therefore are represented with "N/A"

[c] copies/µl

[d] P values are result of a generalized linear model with negative binomial link function using the target response inoculated alone as the reference

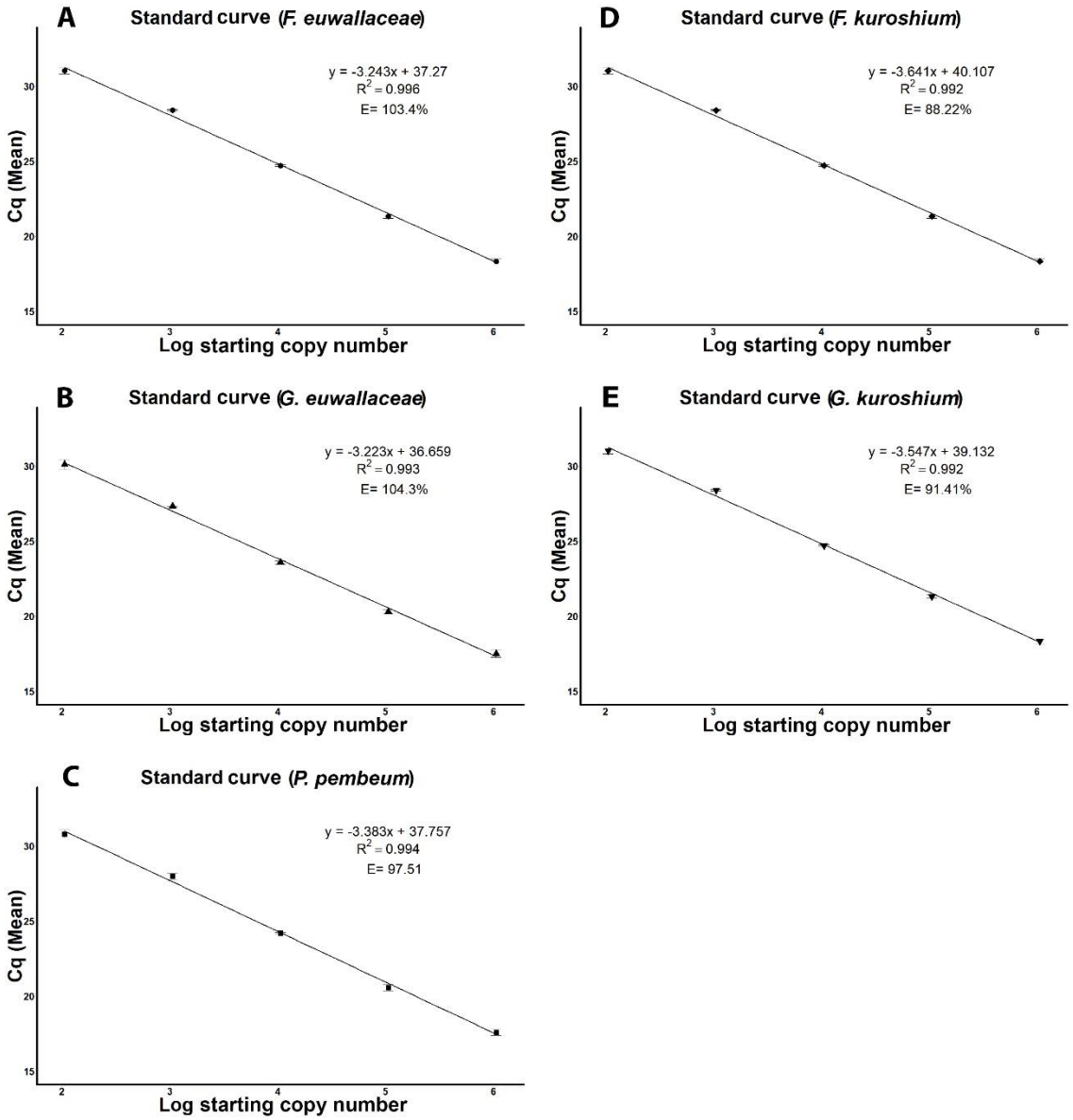

Figure 1.1. Standard curves for qPCR using species specific primers to detect fungi from PSHB (A-C) and KSHB (D-E). The standard curves were constructed using 10-fold dilutions of synthesized dsDNA gBlocks fragments for each target from a starting quantity of $1x10^6$ to $1x10^2$ copies/rxn. Each reaction was done in triplicate efficiencies greater than 88.2 and coefficient of determination greater than 0.992.

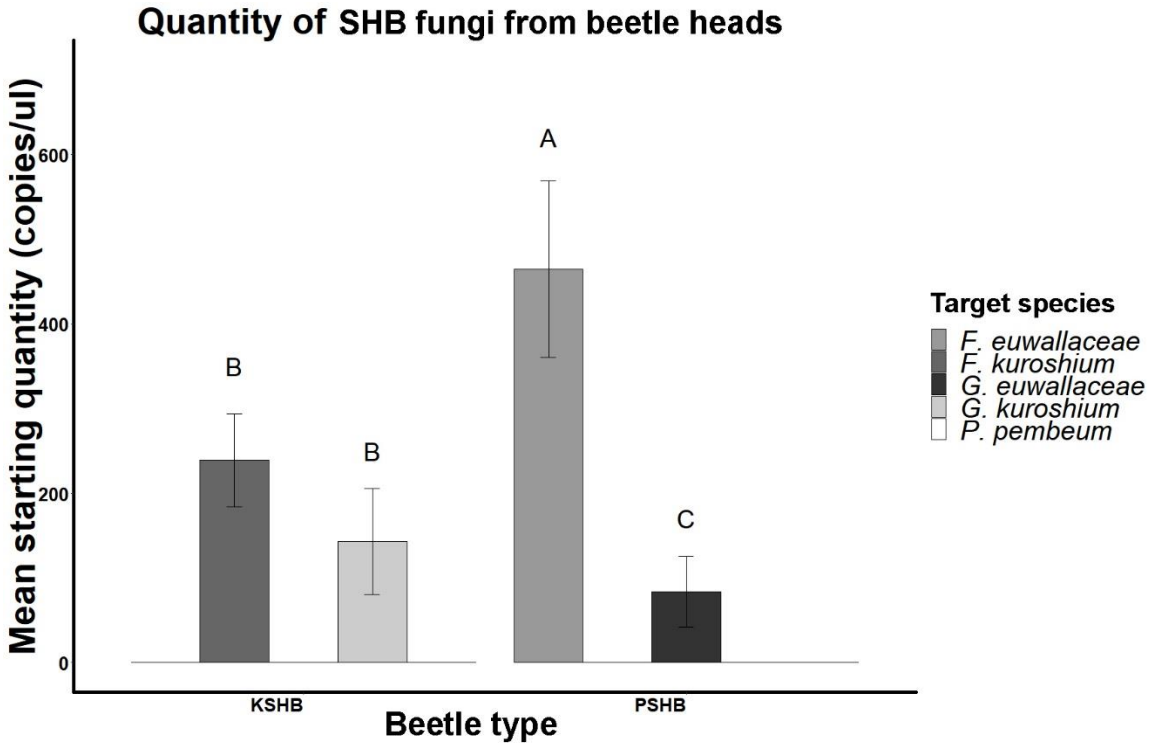

Figure 1.2. Mean starting quantity of fungal symbionts recovered from DNA extracts of female KSHB and PSHB heads using the multiplex real-time assays. Estimation of starting quantity was generated based on multiplexed standard curves performed on each experimental plate using synthetic dsDNA gblocks from each target species from $1x10^6$ to $1x10^2$ copies/rxn. Vertical lines represent standard error of the mean and *P*–values were generated using negative binomial regression analysis and post-hoc analysis from user defined contrasts using the R package "multcomp".

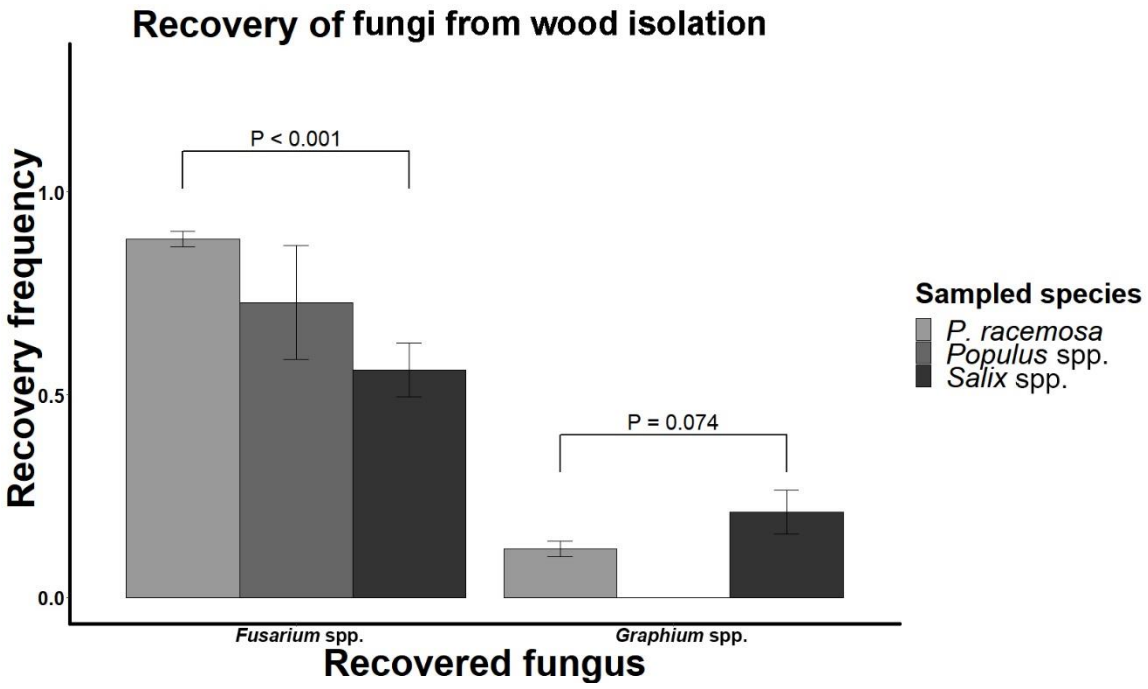

Figure 1.3. Recovery frequency of *Fusarium* spp. and *Graphium* spp. from *P. racemosa*, *Populus* spp. and *Salix* spp. using conventional fungal isolation methods on PDA amended with 0.01% tetracycline. Vertical lines represent standard error of the mean and *P*–values were generated using logistic regression analysis.

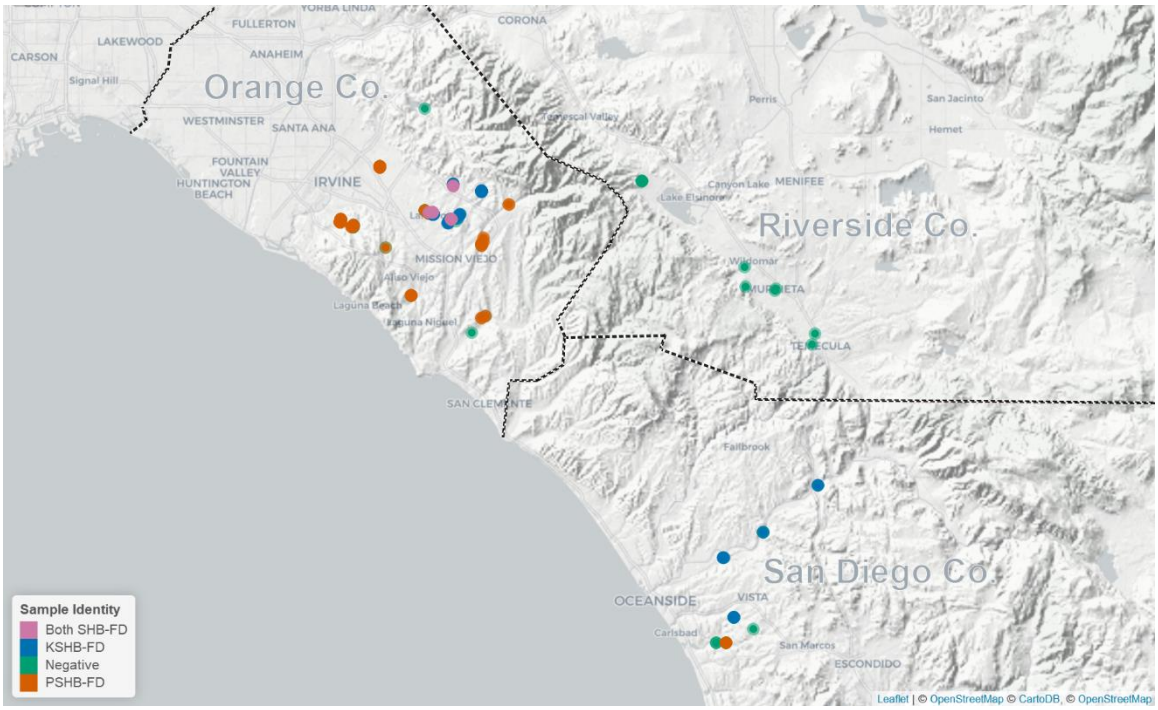

Figure 1.4. Distribution of both PSHB and KSHB from the field sampling across 26 city park locations in Orange, Riverside, and San Diego counties. Diagnosis of the pest-disease complex was done through conventional fungal isolation and real-time qPCR identification of the recovered fungi. PSHB is mainly present in Orange county locations of the map with a single positive location in San Diego County while KSHB is present mainly in San Diego locations but is also present in close proximity to PSHB in Orange County. All points visited in Riverside were negative. The map was generated using the R package “leaflet”.

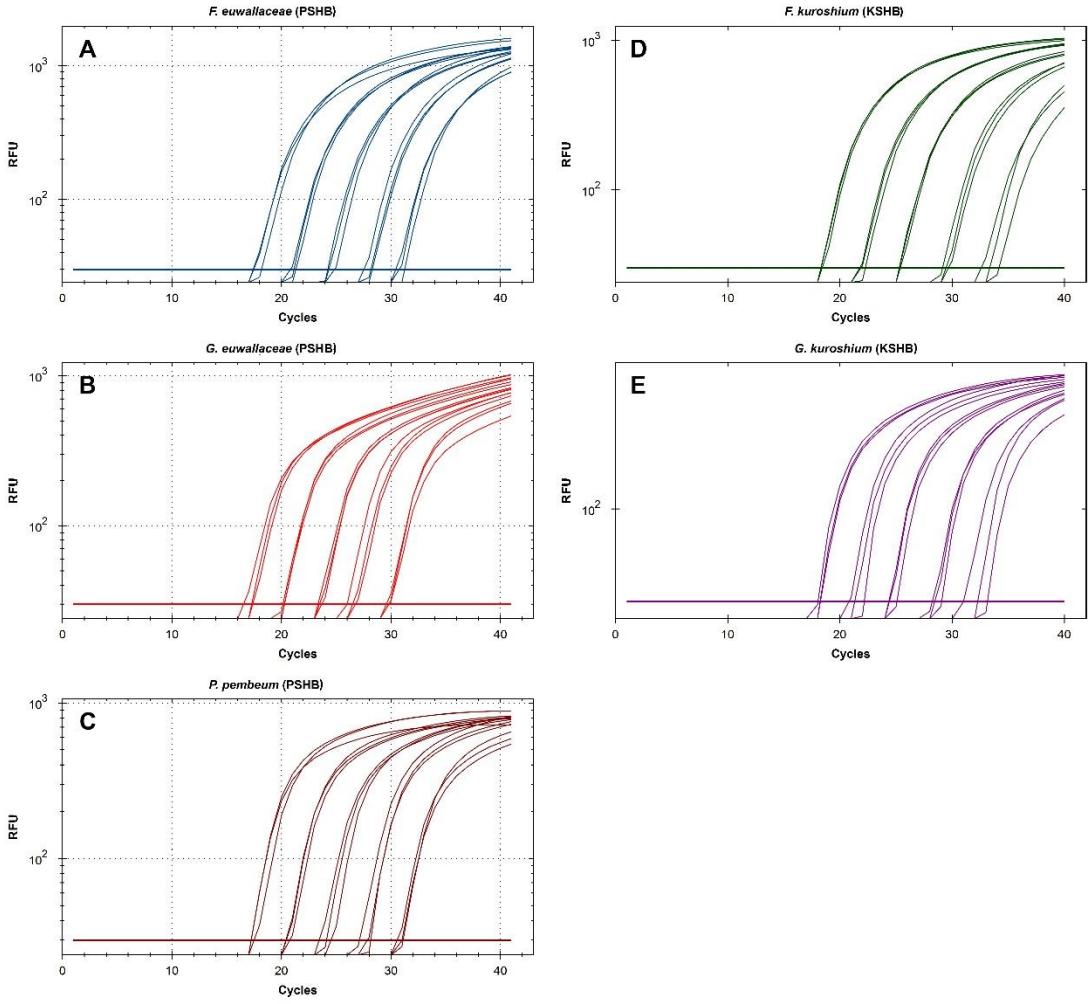

Figure 1.5. Visualization of standard curves for qPCR using species specific primers to detect fungi from PSHB (A-C) and KSHB (D-E). The standard curves were constructed using 10-fold dilutions of synthesized dsDNA gBlocks fragments for each target from a starting quantity of $1x10^6$ to $1x10^2$ copies/rxn. Each reaction was done in triplicate.

CHAPTER II. Members of the Shot Hole Borers *Euwallacea fornicatus* species complex exhibit promiscuous mutualism with ambrosia fungi in Taiwan

ABSTRACT

Ambrosia beetles have been of interest in recent years because of the damage they have been causing in invaded habitats across the globe. Investigation into the nature of members from the *Euwallacea fornicatus* cryptic species complex and their symbioses with fungi in Taiwan revealed promiscuous symbioses with ambrosial Fusaria clade (AFC) members, *Graphium* spp., as well as *Paracremonium* spp. from co-phylogenetic analyses. A novel diagnostic PCR assay targeting mating type genes *MAT1-1-1* and *MAT1-2-1* targeting AFC members was developed and validated by amplicon size and sequencing. Mating types within AFC members were identified and are likely heterothallic with both *MAT* types represented and recovered from *Euwallacea* spp. in Taiwan. Members of the *Euwallacea fornicatus* cryptic species complex and the variety of ambrosia fungi they utilize are more likely promiscuous in nature, as opposed to strictly obligate to a specific suite of fungi as observed in invaded areas.

INTRODUCTION

Bark and ambrosia beetles are well known as forest insects which often cause economic damage to trees and timber (Beaver,1989). Ambrosia beetles in particular (Coleoptera: Curculionidae: Scolytinae) have been of interest in recent years because of the damage they have been causing when invading new habitats in many parts of the world (Carrillo et al. 2014; Eskalen et al. 2013; Mendel et al. 2012; O'Donnell et al. 2016; Hulcr & Stelinski 2016). Although the vast majority of ambrosia beetles have

minimal effect on human industries in natural habitats, there are a few ambrosia beetle species that are serious pests in invaded areas such as *Xyleborus glabratus*, *Xylosandrus* spp., *Euwallacea* spp., *Trypodendron* spp., and *Gnathotrichus* spp. (Carrillo et al. 2014; Hulcr and Stelinski 2016; O'Donnell et al. 2016). These pests rely on symbiotic microorganisms, mainly fungi, as a food source as they tunnel into hardwood trees and cultivate the organisms on nutritionally poor xylem tissue forming fungal gardens inside the colonized host (Beaver, 1989). The fungi associated with the pests are vectored by the beetles within specialized organs termed mycangia (Batra 1963) and can be pathogenic to the plants they colonize and cause damage by inoculating virulent fungi, and or mass inoculation by the pests (Hulcr and Stelinski 2016). For instance, *X. glabratus* with its virulent, primary symbiont *Raffalea lauricola* has killed over a half-billion trees in a decade since its introduction into North America (Hughes, 2013). In California, since 2014, *E whitfordiodendrus* with their symbiotic ambrosia fungi (*Fusarium euwallaceae, Graphium euwallaceae,* and *Paracremonium* pembeum) has infested one-third of California sycamore (*Platanus racemosa*) in Orange County public parks and caused the removal of 1262 trees, resulting in approximately 4 million USD in tree removal costs (OC Parks, 2017).

Euwallacea spp. have been reported to invade multiple areas of the United States including: California, Florida, Hawaii, as well as much of the mid-Atlantic and Southeastern U.S. (CABI 2015; Cognato et al. 2015; Eskalen et al. 2013; Na et al. 2017; O'Donnell et al. 2015; Rabaglia et al. 2006) and other parts of the world including: Australia, Costa Rica, Guatemala, India, Israel, Panama, South Africa, and Sri Lanka

(CABI 2015; Paap et al. 2018; Stouthamer et al. 2017). One the earliest cases of this genera of ambrosia beetles causing economic damage on a plant host was *Euwallacea fornicatus* Eichoff, commonly known as the tea shot hole borer (TSHB), which is associated with *Fusarium ambrosium* and is found in India and Sri Lanka (Danthanarayana, 1968), where it is a serious pest of tea (*Camellia sinensis*). In California (Eskalen et al. 2013; Lynch et al. 2016), Israel (Mendel et al. 2012), and South Africa (Paap et al. 2018) *E. whitfordiodendrus* (Schedl 1942), known as polyphagous shot hole borer (PSHB) has invaded and is associated with *Fusarium euwallaceae* S. Freeman, Z. Mendel, T. Aoki & O'Donnell, *Graphium euwallaceae* M. Twizeyimana, S.C. Lynch & A. Eskalen, and *Paracremonium pembeum* S.C. Lynch & Eskalen, has a vast host range, and is a significant threat to urban landscape, natural habitats, and the avocado industry (Eskalen et al. 2013). Another closely related species *E. kuroshio* (Gomez and Hulcr 2018), known as the Kuroshio shot hole borer (KSHB), was also reported in California (Na et al; 2017; Stouthamer et al. 2017), is associated with *Fusarium kuroshium* F. Na, J. D. Carrillo & A. Eskalen and *Graphium kuroshium* F. Na, J. D. Carrillo & A. Eskalen and thought to have a similar host range to PSHB. The *Fusarium* spp. associated with *Euwallacea* spp. have been referred to as the ambrosia Fusaria clade (AFC) with novel species being numbered (Kasson et al. 2013; O'Donnell et al. 2015). These invasive ambrosia beetles belonging to the *E. fornicatus* species complex are native to southeast Asia (Beaver, 1989; Hulcr and Stelinski 2016; Stouthamer et al. 2017) with recent studies finding additional novel species of AFC members within clade "B" (Kasson et al. 2013) from native areas, such as Taiwan (Na et

al. 2017). Previous sampling and identification at the cytochrome oxidase I (COI) locus in the Taiwan region found TSHB, PSHB, and KSHB haplogroups present (Stouthamer et al. 2017). However,AFC species from Taiwan, or other fungal symbionts like *Graphium* spp. or *Paracremonium* spp., have not been linked to the *Euwallacea* spp. host they were acquired from in previous studies.

Sexual recombination in plant pathogenic *Fusarium* spp. has been described for the *F. graminarium* species complex (teleomorph *Gibberella zeae*, Bowden and Leslie 1999), *F. fujikuroi* species complex (teleomorph *G. fujikuroi*, Kuhlman 1982), and *F. solani* species complex (teleomorph *Nectria haematococca*, Booth 1960), with the latter being the species complex that includes AFC members vectored by *Euwallacea* species. Mating type in *Fusarium* spp. is controlled by a single locus (*MAT)* with two idiomorphic alleles, termed MAT1-1 and MAT1-2 (Kim et al 2012; Leslie & Summerell, 2008). Fungal mating systems in *Fusarium* spp. can be homothallic (self-fertile), as seen in the *F. graminarium* species complex where strains can carry both MAT1-1 and MAT1-2 idiomorphs, or heterothallic (self-sterile) as seen with strains of *F. fujikuroi*, which carry only one mating type (Yun et al. 2000). Members in the *F. solani* species complex have been reported to contain species with heterothallic and homothallic members as well as species with no known sexual stage, which was initially hypothesized for the *F. ambrosium* associated with TSHB (O'Donnell 2000). Mating types of *Fusarium* spp. can be identified using PCR assays which specifically target conserved MAT1-1 (α-BOX) and MAT1-2 (HMG BOX) regions of *Fusarium* spp. to amplify mating type idiomorphs of both sexually (Steenkamp et al. 2002) and asexually reproducing species (Kerenyi et

al. 2004), including some *F. solani* species complex members. Currently, *MAT* genes have not been extensively explored with AFC members associated with *Euwallacea* spp. nor has an assay been validated by PCR amplification of *MAT* regions in strains within the AFC.

It has been traditionally assumed that ambrosia beetles are mostly associated with a single dominant fungus (Kostocvik et al. 2015). However, it has been shown that they may feed on more than one species in fungal galleries (Batra 1966) and the proportion of fungal species in individuals can shift during beetle development and maturation in PSHB (Freeman et al. 2016). Lateral transfer of fungal symbionts has been reported from ambrosia beetles colonizing the same host (Carrillo et al. 2014) and in sympatric regions (Kostocvik et al. 2015). In addition, mycobiome communities surveyed from ambrosia beetle mandibular mycangia exhibit a greater species diversity compared to mesonotal mycangium (Kostocvik et al. 2015). Past research on *Euwallacea* spp. cryptic species complex and their fungal symbionts within their mandibular mycangia have been generally focused on invasive members (Eskalen et al. 2013; Kasson et al. 2013; Mendel et al. 2012; O'Donnell et al. 2015) and it has even been suggested that PSHB and TSHB are strictly obligate to their AFC fungi, *F. euwallaceae* and *F. ambrosium*, respectively (Freeman et al. 2013). The ability of symbiotic fungi to be laterally transferred has limited investigation in native areas like Taiwan, even though promiscuous symbiosis has been reported in many other ambrosia beetle species in invaded areas like Florida (Carrillo et al. 2014; Kostocvik et al. 2015).

The objectives of this study were to investigate beetle-fungi relationships with *Fusarium* spp., *Graphium* spp., and *Paracremonium* spp. recovered from *Euwallacea* spp. in native habitats through identification of the beetle species using real-time quantitative PCR (qPCR) high-resolution melt curve analysis (HRM, Rugman-Jones et al. 2017) coupled with identification of the fungal species recovered from the beetles using multi-gene molecular phylogeny at informative loci. Of the AFC fusaria members, we aimed to develop and validate a PCR assay to investigate mating types present in the population from AFC members recovered from the *Euwallacea* spp. sampled in Taiwan as well as other known AFC species associated with invasive *Euwallacea* spp. The AFC-specific assay can be a useful tool in identify mating types among AFC members introduced and vectored by *Euwallacea* spp. in native and invaded areas throughout the world.

METHODS AND MATERIALS

Sample collection and isolation. Fungal isolates used in this study were obtained from the heads of beetles similar to methods described by Lynch et al (2016) and from wood recovered from *Euwallacea* spp. galleries in their reproductive host trees, similar to methods described by Eskalen et al. (2013). In 2017, Infested avocado (*Persea americana* Mill.) wood branches were shipped to a BSL-2 insectary and quarantine facility at UC Riverside under USDA APHIS permit (P526P-16-03142). From the wood, emerging females were collected to represent a total of 130 *Euwallacea* sp. nr. *fornicatus* specimens from four different locations in the Danei District of Taiwan. The beetles were surface sterilized by submerging in 70% ethanol and vortexed for 20 s, rinsed with sterile

de-ionized water, and allowed to dry on sterile filter paper. Beetle heads were separated from the thoracic and abdominal segments under a dissection microscope, then the head segments were macerated in 1.5 ml microcentrifuge tubes with sterile plastic pestles. The macerated heads were suspended in 1 ml of sterile water and 25 µl of the suspensions were pipetted onto Petri plates containing potato dextrose agar (PDA; BD Difco, Sparks, MD) amended with 0.01% (w/v) tetracycline hydrochloride (PDA-t) and spread using sterile glass L-shaped rods. Plates were incubated for two days at room temperature and single spore fungal colonies with unique morphologies were sub-cultured for further downstream identification. The remaining abdomen/thorax segments were saved for downstream beetle identification to link the fungi to the beetles they came from. In 2018, 40 infested gallery samples from avocado, with live beetles present, were excised with a sterile knife and cut into small pieces that would fit into a 2ml microcentrifuge tube. The beetles that came from the wood were placed into a separate microcentrifuge tube and suspended in 70% ethanol. Both gallery samples and beetles that occupied them were shipped directly to UC Riverside under USDA-APHIS permit (USDA-APHIS AP17PPQS&T00C01). Fungal isolation from wood was performed under a bio-safety cabinet (BSC II) and wood was surface sterilized and plated onto PDA-t petri plates as well as Malachite Green Agar (MGA; Leslie and Summerell 2006) to select for *Fusarium* spp. Plates were incubated at 25°C for five days, and emerging colonies were scraped with a sterile loop, streaked out on PDA-t, and single colonies were isolated after incubation for two days at 25°C. Unique morphologies from *Fusarium* spp., *Graphium* spp., and *Paracremonium* spp. were selected for downstream identification.

DNA extraction, PCR, and phylogenetic analysis. Genomic DNA of the fungal isolates of unique morphology obtained from *Euwallacea* spp. beetles and gallery samples were extracted using a DNeasy plant mini kit (Qiagen, Hilden, Germany). Approximately 25mg of fungal mycelium was harvested from fungal isolates (*Fusarium* spp., *Graphium* spp., and *Paracremonium* spp.) from fully colonized PDA and placed into sterile 1.5 mL microcentrifuge tubes previously loaded with 25µl of AP1 buffer (Qiagen, Hilden, Germany) then frozen at 0°C and macerated with a plastic pestle (Thermo Fisher Scientific, Pittsburgh, PA, USA). Once the tissue was macerated, the DNeasy plant mini kit (Qiagen, Hilden, Germany) manufacturer protocol was used to extract DNA from the samples. All samples were suspended in 50µl AE elution buffer and DNA concentration was quantified using Nanodrop 2000c (Thermo Fisher Scientific, Pittsburgh, PA, USA).

PCR amplification of internal transcribed spacer (ITS)1-5.8S-ITS2 and translation elongation factor 1-α (TEF1-α) was done using ITS4/ITS5 (White et al., 1990) for all species tested, EF1/EF2 for *Fusarium* spp. only (O'Donnell et al. 1998), and EF1F/EF2R for *Graphium* spp. and *Paracremonium* spp. only (Jacobs et al. 2004). In addition PCR amplification of RNA polymerase subunit I (RPB1) and RNA polymerase subunit II (RPB2) loci was performed for *Fusarium* spp. and *Paracremonium* spp. only using primers F5/R8 (RPB1-1) (O'Donnell et al. 2010), F7/G2R (RPB1-2) (O'Donnell et al. 2010), 5F2/7CR (RPB2-1) (O'Donnell et al. 2007), 7CF/11AR (RPB2-2) (O'Donnell et al. 2007). Two additional genes were sequenced for *Paracremonium* spp. including calmodulin (cal) using CAL-228F and CAL2Rd (Carbone and Kohn, 1999) and ATP

citrate lyase region (acl1) with acl1-230up and acl1-1220low primers (Gräfenhan et al. 2011). It should be noted that LSU domains D1 and D2 of the LSU rDNA were previously found to be least informative (Kasson et al. 2013, O'Donnell et al. 2015) therefore were not included in this study. Each PCR reaction mixture consisted of 12.5 µl GoTaq DNA Polymerase (Promega, Madison, WI), 9.3 µl sterile DNase-free water, 0.6 µl of 10 µM forward primer, 0.6 µl of 10 µM reverse primer PCR, and 2 µl of 10ng genomic DNA template to a total of 25 µl reaction mixture. PCR was performed for each primer set using published cycling parameters (Carbone and Kohn, 1999; Groenewald et al., 2013; Jacobs et al. 2004; O'Donnell et al. 1998; O'Donnell et al. 2007; White et al. 1990). Amplified products were separated by gel electrophoresis in 1% agarose gel with 0.5x Tris-boric acid-EDTA buffer, stained with SYBR Green (Invitrogen, Carlsbad, CA), and viewed under UV light. Products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) then sequenced in both directions (Sanger ABI3730) at the Institute for Integrative Genome Biology, University of California Riverside with corresponding primers used for PCR. Raw sequences were assembled in Sequencher 4.6 (Gene Codes Corp., Ann Arbor, MI).

Extraction of genomic DNA from *Euwallacea* spp. samples was done with a rapid extraction protocol and real-time quantitative PCR using high resolution melt curve analysis as described by Rugman-Jones and Stouthamer (2017) to type the beetles as PSHB, KSHB, or TSHB groups. A select group of interest were further sequenced at the COI locus to identify the beetle haplogroups within the *Euwallacea* cryptic species complex described by Stouthamer et al. (2017) in order to link the fungal species

recovered from the sampled beetles. Haplogroups were not used for investigating association of *Graphium* spp. and *Paracremonium* spp.

Multi-gene phylogenetic analysis was conducted to determine the genetic relatedness of *Fusarium* spp., *Graphium* spp., and *Paracremonium* spp. isolates obtained from sampled locations in Taiwan. These isolates were also compared to other members of their respective genera. Phylogenetic analysis of *Fusarium* spp. was conducted using concatenated DNA sequences at ITS, TEF1-α, RPB1, and RPB2 gene regions from thirty-two isolates of *Fusarium* spp. from Taiwan (Table 2.1), along with DNA sequences obtained from GenBank (Table 2.2) from thirty-six isolates previously used in AFC phylogenetic analysis from Kasson et al. (2013) and O'Donnell et al. (2015). Phylogenetic analysis of *Graphium* spp. was conducted using concatenated DNA sequences of ITS and TEF1-α gene regions from twelve isolates of *Graphium* spp. (Table 2.1) as well as twenty-eight isolates obtained from GenBank (Table 2.2) used in a previous analysis by Lynch et al. (2016). Phylogenetic analysis of *Paracremonium* spp. was conducted using concatenated DNA sequences of ITS, TEF1-α, RPB2-1, cmd, and acl1 gene regions from eight isolates of *Paracremonium* spp. (Table 2.1) as well as eleven isolates obtained from GenBank (Table 2.2) used in a previous analysis by Lynch et al. (2016). All respective sequences from *Fusarium* spp. *Graphium* spp., and *Paracremonium* spp. were aligned using Clustal X (Thompson *et al.,* 1997) and concatenated after alignment. A partition file was created to indicate the range of each gene in the concatenated alignment and allow for different substitution models. The multigene phylogenies were constructed with Maximum Likelihood (ML) methods using

IQ-TREE (Nguyen et al. 2015). The ModelFinder option (Kalyaanamoorthy et al., 2017) was used to determine that the best partitioning scheme with the TESTMERGE command (Chernomor et al. 2016) was TEF, and ITS+ RPB1+RPB2 for *Fusarium* spp., ITS+EF for *Graphium* spp., and ITS,TEF,RPB2,ACL,CAL for *Paracremonium* spp. identified the best substitution model for each of the defined partitions (-m MFP -nt AUTO -spp partitions.txt –bb 1000) of *Fusarium* spp., for each of the two defined partitions (-bb 1000 -m TESTNEW -q partitions.txt) of *Graphium* spp., and for five defined partitions (-m MFP -nt AUTO -spp partitions.txt –bb 1000) of *Paracremonium* spp.. The best model to use was TIM3e+I+G4 (ITS+RPB1+RPB2) and K2P+G4 (EF) for *Fusarium* spp., K2P+I (ITS+EF) for *Graphium* spp., and TNe+G4 (ITS), TNe (TEF), TIM3e+G4 (RPB2), K2P+I (acl1), HKY+F+I+G4 (cal) for *Paracremonium* spp. IQ-TREE was run with 1000 standard bootstrap analyses to generate final tree run.

Cophylogenetic analysis. Evidence for cophylogeny between *Euwallacea* spp. and their symbiotic fungi was analyzed separately for *Fusarium* spp., *Graphium* spp., and *Paracremonium* spp. Resultant IQ-TREE ML tree files for each group were directly compared to phylogenies for *Euwallacea* spp. built from sequences described in O'Donnell et al. (2015) were used as input to build distance matrices. Host-symbiont matrices were manually created using the identities of the fungi and which *Euwallacea* spp. they were recovered from. and included in the cophylogenetic test using "parafit" function (Legendre et al. 2002) within the package "ape" (Paradis et al. 2004) in R version 3.4.3 (Team 2013). In parafit analysis, the distance matrices from the *Euwallacea* spp. and symbiotic ambrosia fungi were computed from the generated ML trees.

Probabilities were based on 999 permutations and the correlation was considered significant at $P < 0.05$. The null hypothesis of the global test is that the associations between *Euwallacea* spp. and their symbiotic ambrosia fungi are randomly distributed on the phylogeny. The null hypothesis for individual links is *Euwallacea* spp. and symbiotic ambrosia fungi association is established at random.

Primer design and validation for mating types genes of AFC fusaria. Available genomes from AFC species (GenBank Accession: NHTE00000000, NKUJ00000000, NIZV00000000, NKCL00000000, NKCK00000000, NKCJ00000000, NKCI00000000) were utilized to design novel primers that can identify AFC mating types (MAT) from both AFC clade A and B (Kasson et al. 2013; O'Donnell et al. 2015)) from extracted and aligned sequences from both MAT 1-1-1 and MAT 1-2-1. Conserved regions both MAT 1-1-1 and MAT 1-2-1 genes were selected and melting temperatures and secondary structures of generated oligonucleotides were evaluated using Primer3 (v. 0.4.0, Koressaar and Remm, 2007; Untergasser et al. 2012). Candidate oligonucleotide sequences were also checked for specificity by using Fasta36 (Pearson 2016) against AFC genomes using the -fasta36 tool. The MAT regions used for AFC_MAT1 primers was a partial region from the MAT 1-1-1 gene, while AFC_MAT2 primers were designed around a partial region of the MAT 1-2-1 gene (Table 2.4). The primers were optimized using a gradient. The PCR reactions were carried out in 25µl reactions using GoTaq Green (Promega, Madison, WI) 250nm of each primer set, and 1µl DNA template (at 10ng/ul) or nuclease free water (Thermo Fisher Scientific, Pittsburgh, PA, USA) for non-treated controls (NTC). The optimal conditions for all primer sets was determined to be a

three step protocol: 95°C for 2 m followed by 30 cycles of denaturation at 95°C for 30 s followed by annealing at 60°C for 30 s followed by extension at 72°C for 30 s and a final extension at 72°C for 5 m by using conventional PCR thermal gradient option with a MyCycler® (Bio-Rad, Hercules, CA, USA). End-point analysis of amplified products was done by agarose gel electrophoresis in 1% agarose gel with 0.5x Tris-boric acid-EDTA buffer, stained with SYBR Green (Invitrogen, Carlsbad, CA), and visualized under UV light. Products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) then sequenced in both directions at the Institute for Integrative Genome Biology, University of California Riverside with corresponding primers used for PCR. Raw sequences were assembled in Sequencher 4.6 (Gene Codes Corp., Ann Arbor, MI). Products were validated by using basic local alignment search tool (BLAST, Altschul et al. 1990) against GenBank accessions from closely related *Fusarium* spp. and submitted to GenBank (Table 2.1).

RESULTS

Phylogenetic diversity of fungal symbionts associated with *Euwallacea* spp. in Taiwan. For phylogenetic trees using *Fusarium* spp., sequences of *Fusarium neocosmosporiellum* (NRRL 22468, NRRL 43467) and *F. lichenicola* (NRRL 32434) were used as outgroups for rooting the *Fusarium* tree (Fig 2.1) based on prior analyses of AFC members (Kasson et al. 2013; O'Donnell et al. 2015, Na et al. 2017). Multi-locus phylogenetic analysis performed on four loci (ITS, TEF1-α, RPB1, and RPB2) from *Fusarium* isolates tested in this study indicate *Fusarium* spp. recovered from *Euwallacea* spp. in Taiwan represent AFC species ([AF-13]-[AF-16]) found from Taiwan isolates

(Fig. 2.1) previously (Na et al. 2017) and reside in AFC species major clade B (Kasson et al. 2013; O'Donnell et al. 2015). Interestingly, the AFC species from Taiwan are paraphyletic, with AF-16 forming a monophyletic clade that is sister to AF-[2-4;12-15]. With more isolates representing AF-16 compared to Na et al. (2017), two additional sister clades were resolved within AF-16, including AF-17 and AF-18 (Fig 2.1).

Sequences from previous phylogenetic analysis of *Graphium* spp. (Lynch et al. 2016) were used to build a maximum likelihood phylogenetic tree through multi-locus phylogenetic analysis using two informative loci, ITS and TEF1-α including *Graphium* spp. recovered from *Euwallacea* spp. in Taiwan (Fig 2.2, 2.3). Two isolates (UCR 5548, 5528) recovered from PSHB in Taiwan were found to form a well-supported clade (92% bootstrap support) within the *G. euwallaceae* clade, while all other isolates recovered from PSHB in Taiwan resolving within the *G. kuroshium* clade (Fig 2.2. 2.3) which was initially described when recovered from KSHB in California (Na et al. 2017). *Graphium* spp. recovered from TSHB in Florida formed their own distinct clade (81 % bootstrap support) sister to the *G, euwallaceae* clade and the *G. carboniaruim* clade. In addition to *Graphium* spp., *Paracremonium* spp. were also found associated with *Euwallacea* spp. in Taiwan resolving in *Paracremonium* sp. I clade (Fig 2.4) which include Vietnam isolates recovered from *Euwallacea* sp. initially described by Lynch et al. (2016). *Paracremonium* spp. recovered from TSHB invading Florida and KSHB invading California were also included in this analysis and the former was found to resolve in the *Paracremonium* sp. I clade, similar to the Taiwan isolates, while the latter resolved in the *P. pembeum* clade which was recovered from PSHB in California.

Fungal symbiont promiscuity in *Euwallacea* spp. in Taiwan. By typing the AFC Fusaria and the *Euwallacea* sp. haplotype (Stouthamer et al. 2017) they were recovered from, symbiont fidelity was shown to not be restricted to a specific *Euwallacea* sp. sampled in Taiwan. Cophylogenetic parafit analysis of the *Euwallacea* spp and AFC Fusaria ML trees revealed the associations between the host and symbiont were randomly distributed from the global test results ($P = 0.72$) as well as individual links ($P > 0.05$). Surveying the beetles from wood logs in Taiwan we found PSHB (H38, H22) to be a large representative of each sampling effort, with KSHB (H20) and TSHB (H8) less represented from random sampling of females from the wood (Table 2.4). Out of the 6 AFC species found in Taiwan, KSHB (H20), PSHB (H22), PSHB (H38), and TSHB (H8) were found to vector AF-18 species, PSHB (H22), PSHB (H38) and TSHB (H8) were found to both vector AF-17, AF-16. PSHB (H38) and TSHB (H8) were found to be associated with AF-13 and AF-14, while only PSHB (H38) was found associated with AF-15 and *F. kuroshium* from our sampling efforts in 2017 and 2018 (Fig 2.1). Of the *Graphium* spp. recovered from *Euwallacea* spp. in Taiwan, similar species from the *G euwallaceae* and *G. kuroshium* clades were found to be associated with PSHB and unique species clade was found associated with TSHB in Florida (Fig. 2.2) Cophylogenetic parafit analysis of the *Euwallacea* spp and *Graphium* spp. ML trees revealed the associations between the host and symbiont were randomly distributed from the global test results ($P = 0.14$) but individual link association of *Graphium* sp. I from *E. validus* was found to be non-random ($P= 0.03$) while the other individual link associations *were* random ($P >0.05$. PSHB from Taiwan were found to be associated with *G. kuroshium*, a

species clade closely related to *G. kuroshium*, as well as a species sister to *G. euwallaceae*. *Paracremonium* spp. from the *Paracremonium* sp. I clade (Lynch et al. 2016) were found to be vectored by both PSHB and TSHB in Taiwan while isolates from Florida recovered from TSHB were also within this clade (Fig 2.4). Cophylogenetic parafit analysis of the *Euwallacea* spp and *Paracremonium* spp. ML trees revealed the associations between the host and symbiont were randomly distributed from the global test results ($P = 0.18$) as well as individual links ($P > 0.05$). No species recovered from *Euwallacea* spp. in Taiwan resolved in the *P. pembeum* clade which is vectored by PSHB invading California except for the *Paracremonium* spp. recovered from KSHB in California.

AFC mating type specific PCR assay or mating type specific PCR assay to distinguish AFC in Taiwan or PCR based assay to detect AFC in Taiwan. The degenerate primers (Table 2.4) for the novel PCR assay for screening *MAT* type targeting AFC members was found to produce products in all AFC isolates tested including AF [1-2]; AF-[4-6]; AF-[8-18] (Table 2.5). AFC members were found to be heterothallic with all isolates tested either showing product for *MAT1-1-1* or *MAT1-2-1*. The invasive AFC species *F. euwallaceae* [AF-2] and *F. kuroshium* [AF-12] appear to be both MAT1-1 while *Fusarium* spp. [AF-6] in Florida has both mating types present in the population. Product size for *MAT1-1-1* was approximately 550 bp for AF-2 (*F. euwallaceae)* and AF-12 (*F. kuroshium*) and 600 bp for Taiwan isolates, while *MAT1-2-1* for Taiwan isolates was 800 bp (Fig 2.5). Sequences obtained from isolates validated *MAT* products as *MAT1-1-1* or *MAT1-2-1* from *in silico* BLAST results from closely related species (data

not shown). Within promiscuous AFC species (AF-[13,14,16-18]) isolates included in the phylogenetic study, *Euwallacea* spp. in Taiwan were found to vector different AFC species while also vectoring both mating types *within* the AFC species (Fig 2.1). Within sampled regions in Taiwan, both mating types were represented among the sampled regions (Table 2.3).

DISCUSSION

Investigation into the nature of members from the *Euwallacea fornicatus* cryptic species complex (PSHB, TSHB, and KSHB) and their symbioses with fungi in Taiwan revealed promiscuous symbioses with AFC members, *Graphium* spp., as well as *Paracremonium* spp. from co-phylogenetic analyses (Figs 2.1, 2.2, 2.3, 2.4). *Euwallacea* spp. in invaded areas have been initially reported to be associated exclusively with specific AFC members (Freeman et al. 2013; O'Donnell et al. 2015) as well as *Graphium* spp. and *Paracremonium* spp. (Lynch et al 2016; Na et al. 2017), where in native habitats like Southeast Asia, *Euwallacea* spp. likely have a less exclusive relationship with the ambrosia fungi they vector based on the results from this study. In addition, AFC members appear to be heterothallic (Fig 2.5); with both *MAT* types represented and recovered from *Euwallacea* spp. inhabiting host wood in the native habitat (Table 2.3). The diagnostic assay targeting *MAT* types may be a useful diagnostic tool to type invasive AFC members associated with *Euwallacea* spp. to investigate if both idiomorphs are present in invaded areas and if mating is occurring in said areas. Members of the *Euwallacea fornicatus* cryptic species complex and the variety of ambrosia fungi they

utilize are more likely promiscuous in nature, as opposed to strictly obligate to a specific suite of fungi as observed in invaded areas.

The AFC species recovered from *Euwallacea* spp. samples in this study represent the same species clades described from previously reported Taiwan AFC isolates (Na et al. 2017), which found new AFC species AF-13 to AF-16. Our sampling efforts from this study discovered more isolates within the AF-16 clade which diverge to 3 strongly supported clades which we are terming AF-16 (98% bootstrap), AF-17 (90% bootstrap), and AF-18 (98% bootstrap) (Fig 2.1) determined using a multi-gene phylogeny. Interestingly, UCR6408 was identical to *F. kuroshium* (AF-12) associated with KSHB in California, however it was recovered from PSHB mycangia in this study. All of the *Graphium* spp. recovered from *Euwallacea* spp. in this study were found to be closely related to either *G. euwallaceae* or *G. kuroshium* (Fig 2.2, 2.3). Two isolates UCR5519 and UCR5549 were found to be identical to *G. kuroshium* associated with KSHB in California, but again was recovered from PSHB mycangia as opposed to KSHB in California. The finding of *F. kuroshium* and *G. euwallaceae* corroborates the findings of Stouthamer et al. 2017 that Taiwan may be an origin of invasive KSHB-FD in California. *Paracremonium* spp. found from beetles in Taiwan and Florida did not clade with *P. pembeum* associated with PSHB in California, but instead *Paracremonium* sp. I previously found in Vietnam (Fig. 2.4) (Lynch et al. 2016). We also are reporting a *Paracremonium* spp. recovered from KSHB infested plants in California that is closely related to *P. pembeum* which was not reported as a KSHB symbiont by Na et al. 2017. These fungi are not recovered frequently from female mycangia, but rather beetle larvae

and gallery samples (Freeman et al. 2015; Lynch et al. 2016). There also can be significant difference in the proportion of these fungal propagules in isolation plating from different attacked hosts (Lynch et al. 2016). However, the function of this group is still unknown, but recovering these members from a native area such as Taiwan provides more evidence that the presence of these fungi may be important in ambrosia fungi symbiosis. The presence auxiliary fungi such as *Graphium* spp. and *Paracremonium* spp. possibly contribute to the polyphagous habits of these ambrosia beetles and allow colonization of a wide range of hosts which has been reported in other ambrosia beetle systems (Batra 1966, Beaver 1989, Nakashima 1987).

Ambrosia beetles from the *Euwallacea* genus have invaded the non-native areas on multiple occasions (CABI 2015; Eskalen et al. 2013; Hulcr and Dunn 2011, Hulcr and Stelinski 2016; Kasson et al. 2013; Mandel et al. 2012; O'Donnell et al. 2015; Paap et al. 2018; Stouthamer et al. 2017) and carry fungal symbionts that have evolved with them in mutualisms that provide the beetles with nutritional supplement from low-nutrient xylem tissue, while the fungi receive reliable dispersion and direct inoculation into plant hosts (Beaver, 1989). The present study suggests a non-exclusive relationship exists between *Euwallacea fornicatus* cryptic species complex members and closely related fungal species from specific genera: *Fusarium* spp. (AFC), *Graphium* spp., and *Paracremonium* spp., which may all support nutritional requirements for the lifestyle of the insect vector and different stages of development (Freeman et al. 2013; Freeman et al; 2016). It is clear that AFC members, such as *F. euwallaceae* associated with PSHB, are the most prevalent in female mycangia (Kasson et al. 2013; Lynch et al. 2016), most likely to be recovered

from gallery samples (Carrillo, Mayorquin, and Eskalen, *Unpublished data*), and lead to increased fecundity as a diet source over auxiliary fungi *G. euwallaceae* and *P. pembeum* in diet studies Freeman et al. 2013; 2016). It was initially hypothesized that *Euwallacea* spp., such as PSHB, require their specific *Fusarium* associates to survive and reproduce (Freeman et al. 2013), but here we show evidence that the relationship is nonspecific to a particular AFC species and is occurring in their native habitat in Taiwan with TSHB and PSHB both found vectoring multiple AFC species present in Taiwan (Fig 2.1). The reason the previous diet studies may have led to such results, is the beetles were introduced to the AFC member in the larval stage instead as opposed to using aseptic virgin female adults as done in early experiments dealing with ambrosia beetles (Batra 1966). In a later diet experiment (Freeman et al. 2016), *G. euwallaceae* was found present significantly more in larvae and hypothesized to reduce competition between adults and developing larvae, but they could not survive or reproduce on *P. pembeum* alone. To investigate function of the ambrosia fungi, future studies should consider the life stages in which the beetles are being introduced experimentally and test if promiscuous symbiosis is possible in areas with multiple invasive *Euwallacea* spp. such as California and Florida in the United States.

The invasion of ambrosia beetles is a growing concern due to the ability of some invaders to attack and colonize healthy hosts with aid of their symbiotic partners. Of these invasive species *X. glabaratus* causing the disease Laurel wilt is of concern because of the virulent nature of the plant pathogen *Raffaelea* spp. they vector which has led to the destruction of an estimated half billion native trees in affected regions (Hughes 2013).

Similarly, *Euwallacea* spp. can attack and inoculate their plant pathogenic mutualists into healthy and declining trees (Hulcr and Stelenski 2016) and pose a threat to native, urban, and agricultural landscapes (Eskalen et al. 2013; O'Donnell et al. 2016). Plant pathogenic fungi can recombine to combat selective pressures such as host resistance genes and fungicide application, which has been reported in *Puccinia* spp. on wheat (Kim and Brewmaker 1977) as well as in *F. gramanarium* on wheat (Miedaner et al. 2001). When a compatible mating partner is introduced into areas with a clonal linage the result can yield higher virulence and resistance to host defenses and the rise of new clonal populations. We have shown here that native populations of *Euwallacea fornicatus* cryptic species complex members are vectoring different mating types of the heterothallic AFC species acquired from the host (Fig 2.1, 2.5; Table 2.3, 2.5). Of invasive AFC members, *F. euallaceae* and *F kuroshium* (AF-12), only one clonal population with mating type *MAT1-1-1* is present in California where *Fusarium* spp. (AF-6) in Florida has both mating types present within the population based on the results from this study. The introduction of new mating types into areas into already affected areas may lead to increased diversity if these beetles and their mutualist fungal species encounter a new clonal lineage with a compatible mating type, which may be occurring in Florida since both *MAT1-1* and *MAT1-2* mating types are already present (Table 2.5). It should also be investigated if sexual recombination is indeed possible through sexual crosses within AFC species (and possibly *Graphium* spp. and *Paracremonium* spp) and the effects such events have on the vector. A diagnostic assay that can target the *MAT* genes of invasive

AFC members provides valuable tool in identifying the mating types of AFC species in existing and new invasions of *Euwallacea* spp.

As researchers explore deeper into ambrosia beetle symbiosis, the fungal-insect interactions continue to present more complexities as there are now potential fungal-fungal interactions with, at least, AFC Fusaria in these systems with potential for mating. How and if the ambrosia beetles have abilities to control this interaction or simply just survive with what suite of fungi they happen to encounter remains to be seen. It is apparent that other ambrosia beetle systems are promiscuous with their fungi (Carrillo et al 2014; Hulcr and Cognato 2010; Kostovcik et al. 2015; Skelton et al. 2019), while some are apparently more specific (Batra 1985; Beaver 1989).The physical and/or molecular mechanisms which the ambrosia fungi are accumulated and purified in the mycangia should be further investigated to potentially find a weakness to exploit for control in invaded areas. The promiscuous nature of members of the *E. fornicatus* cryptic species complex with their symbiotic ambrosia fungi continues to raise concern in invaded areas, such as California, and the potential for PSHB and KSHB complexes present in California to exchange plant pathogenic fungi and should alert local and government agencies for the effects this can have in an already destructive pathosystem.

LITERATURE CITED

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol., 215(3), 403-410.

Batra, L.R., 1963. Ecology of ambrosia fungi and their dissemination by beetles. Transactions of the Kansas Academy of Science *(1903-)*, 66(2), 213-236.

Batra, L.R., 1966. Ambrosia fungi: extent of specificity to ambrosia beetles. Science, 153(3732), 193-195.

Beaver RA. 1989. Insect–fungus relationships in the bark and ambrosia beetles. In: Wilding N, Collins NM, Hammond PM, Webber JF (eds). Insect–fungus Interactions. Academic Press: London, UK, 121–143.

Booth, C., 1960. Studies of Pyrenomycetes. V. Nomenclature of some Fusaria in relation to their nectrioid perithecial states. Mycological Papers, (74).

Bowden, R.L. and Leslie, J.F., 1999. Sexual recombination in Gibberella zeae. Phytopathology, 89(2), 182-188.

CABI 2015. *Euwallacea fornicatus* Distribution Map

Carbone, I. and Kohn, L.M., 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia, 553-556.

Carrillo, D., Duncan, R.E., Ploetz, J.N., Campbell, A.F., Ploetz, R.C. and Peña, J.E., 2014. Lateral transfer of a phytopathogenic symbiont among native and exotic ambrosia beetles. Plant Pathol., 63(1), 54-62.

Cognato, A.I., Hoebeke, E.R., Kajimura, H. and Smith, S.M. 2015. History of the exotic ambrosia beetles *Euwallacea interjectus* and *Euwallacea validus* (Coleoptera: Curculionidae: Xyleborini) in the United States. Journal of economic entomology, 108(3), 1129-1135.

Danthanarayana, W., 1968. The distribution and host-range of the shot-hole borer (*Xyleborus fornicatus* Eich.) of tea. Tea Quarterly. 39, 61–69.

Eskalen, A., Stouthamer, R., Lynch, S. C., Rugman-Jones, P. F., Twizeyimana, M., Gonzalez, A., & Thibault, T. 2013. Host Range of Fusarium Dieback and Its Ambrosia Beetle (Coleoptera: Scolytinae) Vector in Southern California. Plant Dis., 97(7), 938–951.

Freeman, S., Sharon, M., Maymon, M., Mendel, Z., Protasov, A., Aoki, T., Eskalen, A., O'Donnell, K. 2013. *Fusarium euwallaceae* sp. nov.-a symbiotic fungus of *Euwallacea sp*., an invasive ambrosia beetle in Israel and California. Mycologia, 105(6), 1595–1606.

Freeman, S., Protasov, A., Sharon, M., Mohotti, K., Eliyahu, M., Okon-Levy, N., Maymon, M., Mendel, Z. 2012. Obligate feed requirement of *Fusarium sp*. nov., an avocado wilting agent, by the ambrosia beetle *Euwallacea* aff. *fornicata*. Symbiosis, 58(1-3), 245–251.

Freeman, S., Sharon, M., Dori-Bachash, M., Maymon, M., Belausov, E., Maoz, Y., Margalit, O., Protasov, A. and Mendel, Z., 2016. Symbiotic association of three fungal species throughout the life cycle of the ambrosia beetle *Euwallacea* nr. *fornicatus*. Symbiosis, 68(1-3), 115-128.

Glass, N.L. and Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Environ. Microb., 61(4), 1323-1330.

Gräfenhan, T., Schroers, H.J., Nirenberg, H.I. and Seifert, K.A., 2011. An overview of the taxonomy, phylogeny, and typification of nectriaceous fungi in Cosmospora, Acremonium, Fusarium, Stilbella, and Volutella. Studies in Mycology, 68, 79-113.

Hofstetter, R. W., Dinkins-Bookwalter, J., Davis, T. S., & Klepzig, K. D. 2015. Symbiotic Associations of Bark Beetles. Bark Beetles: Biology and Ecology of Native and Invasive Species. Elsevier Inc.

Hulcr, J., & Cognato, A. I. 2010. Repeated evolution of crop theft in fungus-farming ambrosia beetles. Evolution, 64(11), 3205–3212.

Hulcr, J. and Stelinski, L.L., 2017. The ambrosia symbiosis: from evolutionary ecology to practical management. Annual review of entomology, 62, 285-303.

Hulcr, J., Black, A., Prior, K., Chen, C.Y. and Li, H.F. 2017. Studies of Ambrosia Beetles (Coleoptera: Curculionidae) in Their Native Ranges Help Predict Invasion Impact. Fla. Entomol., 100(2), 257-261.

Hughes MA. 2013. The evaluation of natural resistance to laurel wilt disease in redbay (*Persea borbonia*). PhD. Thesis, Univ. Florida, Gainesville

Jacobs, K., Bergdahl, D. R., Wingfield, M. J., Halik, S., Seifert, K. a, Bright, D. E., & Wingfield, B. D. 2004. *Leptographium wingfieldii* introduced into North America and found associated with exotic *Tomicus piniperda* and native bark beetles. Mycol. Res., 108(April), 411–418.

Kasson, M. T., O'Donnell, K., Rooney, A. P., Sink, S., Ploetz, R. C., Ploetz, J. N., Konkol, J. L., Carrillo, D., Freeman, S., Mendel, Z., Smith, J. A., Black, A. W., Hulcr, J., Bateman, C., Stefkova, K., Campbell, P. R., Geering, A. D. W., Dann, E. K., Eskalen, A., Mohotti, K., Short, D. P. G., Aoki, T., Fenstermacher, K. A., Davis, D. D., Geiser, D. M. 2013. An inordinate fondness for *Fusarium*: Phylogenetic diversity of fusaria cultivated by ambrosia beetles in the genus *Euwallacea* on avocado and other plant hosts. Fungal Genet. Biol., 56, 147–157.

Kerényi, Z., Moretti, A., Waalwijk, C., Oláh, B. and Hornok, L., 2004. Mating type sequences in asexually reproducing Fusarium species. Applied and Environmental Microbiology, 70(8), 4419-4423.

Kim, S.K. and Brewbaker, J.L., 1977. Inheritance of General Resistance in Maize to *Puccinia sorghi* Schw. 1. *Crop science*, *17*(3), 456-461.Koressaar, T. and Remm, M., 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics, 23(10), 1289-1291.

Kostovcik, M., Bateman, C. C., Kolarik, M., Stelinski, L. L., Jordal, B. H., & Hulcr, J. 2015. The ambrosia symbiosis is specific in some species and promiscuous in others: evidence from community pyrosequencing. *ISME J.*, 9(1), 126–138.

Kuhlman, E.G., 1982. Varieties of *Gibberella fujikuroi* with anamorphs in Fusarium section Liseola. Mycologia, 759-768.

Legendre, P., Desdevises, Y. and Bazin, E., 2002. A statistical test for host–parasite coevolution. Systematic biology, 51(2), 217-234.

Leslie, J. F., & Summerell, B. A. 2008. The Fusarium laboratory manual. John Wiley & Sons.

Lynch, S. C., Twizeyimana, M., Mayorquin, J. S., Wang, D. H., Na, F., Kayim, M., ... & Hulcr, J. 2016. Identification, pathogenicity and abundance of *Paracremonium pembeum* sp. nov. and *Graphium euwallaceae* sp. nov.—two newly discovered mycangial associates of the polyphagous shot hole borer (*Euwallacea sp*.) in California. Mycologia, 108(2), 313-329.

Massoumi Alamouti, S., Tsui, C. K. M., & Breuil, C. 2009. Multigene phylogeny of filamentous ambrosia fungi associated with ambrosia and bark beetles. Mycol. Res., 113(8), 822–835.

Mendel, Z., Protasov, A., Sharon, M., Zveibil, A., Yehuda, S. Ben, O'Donnell, K., Rabaglia, R., Wysoki, M., Freeman, S. 2012. An Asian ambrosia beetle *Euwallacea fornicatus* and its novel symbiotic fungus *Fusarium* sp. pose a serious threat to the Israeli avocado industry. Phytoparasitica, 40(3), 235–238.

Miedaner, T., Schilling, A.G. and Geiger, H.H., 2001. Molecular genetic diversity and variation for aggressiveness in populations of *Fusarium graminearum* and *Fusarium culmorum* sampled from wheat fields in different countries. Journal of Phytopathology, 149(11-12), 641-648.

Na, F., Carrillo, J.D., Mayorquin, J.S., Ndinga-Muniania, C., Stajich, J.E., Stouthamer, R., Huang, Y.T., Lin, Y.T., Chen, C.Y. and Eskalen, A., 2018. Two novel fungal symbionts *Fusarium kuroshium* sp. nov. and *Graphium kuroshium* sp. nov. of Kuroshio shot hole borer (*Euwallacea* sp. nr. *fornicatus*) cause Fusarium dieback on woody host species in California. Plant Dis., 102 (6) 1154-1164.

Nakashima, T., Goto, C. and Iizuka, T., 1987. The Primary and Auxiliary Ambrosia Fungi Isolated from the Ambrasia Beetles, *Scolytoplatypus shogun blanford* (Coleoptera, Scolytidae) and Crosstarsus niponicus blandford (*Cleoptera platypodidae*). *Journal of the Faculty of Agriculture,* Hokkaido University= 北海道大學農學部紀要, 63(2), 185-208.

Nguyen, Lam-Tung, Heiko A. Schmidt, Arndt von Haeseler, and Bui Quang Minh. 2015."IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies." Mol. Bio. Evol. 32:1 268-274.

OC Parks 2016. Shot Hole Borer: Managing the Invasive Beetle. https://oc-parks-gis.maps.arcgis.com/apps/Cascade/index.html?appid=680fd0c9e73f4857a8477791f7ee796f.

O'Donnell, K., E. Cigelnik, and H. I. Nirenberg. 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia. 90:465–493.

O'Donnell, K., 2000. Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. *Mycologia*, 919-938.

O'Donnell, K., Sarver, B. A. J., Brandt, M., Chang, D. C., Noble-Wang, J., Park, B. J., Sutton, D. A., Benjamin, L., Lindsley, M., Padhye, A., Geiser, D. M., Ward, T. J. 2007. Phylogenetic diversity and microsphere array-based genotyping of human pathogenic fusaria, including isolates from the multistate contact lens-associated U.S. keratitis outbreaks of 2005 and 2006. J. Clin. Microbiol., 45(7), 2235–2248.

O'Donnell, K., Sutton, D. A., Rinaldi, M. G., Sarver, B. A. J., Balajee, S. A., Schroers, H. J., Summerbell, R. C., Robert, V. A. R. G., Crous, P. W., Zhang, N., Aoki, T., Jung, K., Park, J., Lee, Y. H., Kang, S., Park, B., Geiser, D. M. 2010. Internet-accessible DNA sequence database for identifying fusaria from human and animal infections. J. Clin. Microbiol., 48(10), 3708–3718.

O'Donnell, K., Sink, S., Libeskind-Hadas, R., Hulcr, J., Kasson, M. T., Ploetz, R. C., Konkol, J. L., Ploetz, J. N., Carrillo, D., Campbell, A., Duncan, R. E., Liyanage, P. N., Eskalen, A., Na, F., Geiser, D. M., Bateman, C., Freeman, S., Mendel, Z., Sharon, M., Aoki, T., Crossé, A. A., Rooney, A. P. 2015. Discordant phylogenies suggest repeated host shifts in the *Fusarium-Euwallacea* ambrosia beetle mutualism. Fungal Genet. Biol., 82, 277–290.

O'Donnell, K., Libeskind-Hadas, R., Hulcr, J., Bateman, C., Kasson, M.T., Ploetz, R.C., Konkol, J.L., Ploetz, J.N., Carrillo, D., Campbell, A. and Duncan, R.E., 2016. Invasive Asian Fusarium–Euwallacea ambrosia beetle mutualists pose a serious threat to forests, urban landscapes and the avocado industry. Phytoparasitica, 44(4), 435-442.

Paap, T., De Beer, Z.W., Migliorini, D., Nel, W.J. and Wingfield, M.J., 2018. The polyphagous shot hole borer (PSHB) and its fungal symbiont *Fusarium euwallaceae*: a new invasion in South Africa. Australas. Plant Path., 47(2), 231-237.

Paradis, E., Claude, J. and Strimmer, K., 2004. APE: analyses of phylogenetics and evolution in R language. Bioinformatics, 20(2), 289-290.

Pearson, W.R., 2016. Finding protein and nucleotide similarities with FASTA. Current protocols in bioinformatics, 53(1), 3-9.

Rugman-Jones, P.F. and Stouthamer, R., 2017. High-resolution melt analysis without DNA extraction affords rapid genotype resolution and species identification. Mol. Ecol. Resour., 17(4), 598-607.

Skelton, J., Johnson, A.J., Jusino, M.A., Bateman, C.C., Li, Y. and Hulcr, J., 2019. A selective fungal transport organ (mycangium) maintains coarse phylogenetic congruence between fungus-farming ambrosia beetles and their symbionts. Proceedings of the Royal Society B, 286 (1894), p.20182127.

Steenkamp, E.T., Wingfield, B.D., Desjardins, A.E., Marasas, W.F. and Wingfield, M.J., 2002. Cryptic speciation in *Fusarium subglutinans*. Mycologia, 94(6), 1032-1043.

Stouthamer, R., Rugman-Jones, P., Thu, P.Q., Eskalen, A., Thibault, T., Hulcr, J., Wang, L.J., Jordal, B.H., Chen, C.Y., Cooperband, M. and Lin, C.S., 2017. Tracing the origin of a cryptic invader: phylogeography of the E uwallacea fornicatus (C oleoptera: C urculionidae: S colytinae) species complex. Agricultural and forest entomology, 19(4), 366-375.

Team, R.C., 2013. R: A language and environment for statistical computing.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. 1997. The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res., 25(24), 4876–4882.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M. and Rozen, S.G., 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res., 40(15), 115-115.

Verrall, A. F. 1943. Fungi associated with certain ambrosia beetles. J. Agric. Res. 66: 135-144

White, T.J., Bruns, T., Lee, S.J.W.T. and Taylor, J.L., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 18(1), 315-322.

Yun, S. H., T. Arie, I. Kaneko, O. C. Yoder, and B. G. Turgeon. 2000. Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella*/*Fusarium* species. Fungal Genet. Biol. 31:7–20

Table 2.1. Representative isolates of *Fusarium* spp., *Graphium* spp., and *Paracremonium* spp. from *Euwallacea* spp. and *Persea americana* obtained in this study

				GenBank accession numbers[ab]					
Isolate	**Species**	**Host**	**Location**	**ITS**	**EF1-α**	**RPB1**	**RPB2**	**cmdA**	**acl1**
TW1	*Fusarium* sp.	*Persea americana*	Danei District, Taiwan	MK432860	MK435437	MK435489	MK435521	…	…
TW2	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432862	MK435439	MK435491	MK435523	…	…
TW4	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432866	MK435443	MK435495	MK435527	…	…
TW15	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432861	MK435438	MK435490	MK435522	…	…
TW25	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432863	MK435440	MK435492	MK435524	…	…
TW34	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432864	MK435441	MK435493	MK435525	…	…
TW37	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432865	MK435442	MK435494	MK435526	…	…
TW40	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432867	MK435444	MK435496	MK435528	…	…
TW43	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432868	MK435445	MK435497	MK435529	…	…
TW44	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432869	MK435446	MK435498	MK435530	…	…
TW45	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432870	MK435447	MK435499	MK435531	…	…
TW55	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432871	MK435448	MK435500	MK435532	…	…
TW56	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	MK432872	MK435449	MK435501	MK435533	…	…
UCR5499	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432873	MK435450	MK435502	MK435534	…	…
UCR5508	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432874	MK435451	MK435503	MK435535	…	…
UCR5509	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432875	MK435452	MK435504	MK435536	…	…
UCR5513	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432876	MK435453	MK435505	MK435537	…	…
UCR5545	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432877	MK435454	MK435506	MK435538	…	…
UCR5546	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432878	MK435455	MK435507	MK435539	…	…
UCR5557	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432879	MK435456	MK435508	MK435540	…	…
UCR5584	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432880	MK435457	MK435509	MK435541	…	…
UCR6394	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432881	MK435458	MK435510	MK435542	…	…
UCR6395	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432882	MK435459	MK435511	MK435543	…	…
UCR6403	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432883	MK435460	MK435512	MK435544	…	…
UCR6405	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432884	MK435461	MK435513	MK435545	…	…
UCR6408	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432885	MK435462	MK435514	MK435546	…	…
UCR6409	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432886	MK435463	MK435515	MK435547	…	…
UCR6411	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432887	MK435464	MK435516	MK435548	…	…
UCR6414	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432888	MK435465	MK435517	MK435549	…	…
UCR6417	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432889	MK435466	MK435518	MK435550	…	…
UCR6432	*F. kuroshium*	*Euwallacea* sp.	Danei District, Taiwan	MK432890	MK435467	MK435519	MK435551	…	…
UCR6436	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432891	MK435468	MK435520	MK435552	…	…
UCR5497	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432903	MK435469	…	…	…	…
UCR5501	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432902	MK435470	…	…	…	…
UCR5506	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432901	MK435471	…	…	…	…
UCR5512	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432900	MK435472	…	…	…	…
UCR5517	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432899	MK435473	…	…	…	…
UCR5519	*G. kuroshium*	*Euwallacea* sp.	Danei District, Taiwan	MK432898	MK435474	…	…	…	…
UCR5528	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432897	MK435475	…	…	…	…
UCR5531	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432896	MK435476	…	…	…	…
UCR5548	*Graphium* sp.	*Euwallacea* sp.	Danei District, Taiwan	MK432895	MK435477	…	…	…	…
UCR5549	*G. kuroshium*	*Euwallacea* sp.	Danei District, Taiwan	MK432894	MK435478	…	…	…	…
UCR6662	*Graphium* sp.	*Euwallacea* sp.	FL (USA)	MK432893	MK435479	…	…	…	…
UCR6667	*Graphium* sp.	*Euwallacea* sp.	FL (USA)	MK432892	MK435480	…	…	…	…
TW9	*Paracremonium* sp.	*P. americana*	Danei District, Taiwan	MK432907	MK435484	…	MK435556	MK435572	MK435564
TW19	*Paracremonium* sp.	*P. americana*	Danei District, Taiwan	MK432904	MK435481	…	MK435553	MK435569	MK435561
TW38	*Paracremonium* sp.	*P. americana*	Danei District, Taiwan	MK432905	MK435482	…	MK435554	MK435570	MK435562
TW39	*Paracremonium* sp.	*P. americana*	Danei District, Taiwan	MK432906	MK435483	…	MK435555	MK435571	MK435563
UCR6659	*Paracremonium* sp.	*Euwallacea* sp.	FL (USA)	MK432908	MK435485	…	MK435557	MK435573	MK435565
UCR6671	*Paracremonium* sp.	*Euwallacea* sp.	FL (USA)	MK432909	MK435486	…	MK435558	MK435574	MK435566
UCR7194	*Paracremonium* sp.	*Euwallacea* sp.	CA (USA)	MK432910	MK435487	…	MK435559	MK435575	MK435567
UCR7195	*Paracremonium* sp.	*Euwallacea* sp.	CA (USA)	MK432911	MK435488	…	MK435560	MK435576	MK435568

[a]ITS = internal transcribed spacer region; EF1-α = translation elongation factor 1-α;

[b]RPB = DNA-directed RNA polymerase II subunit; cmdA = calmodulin; acl1 = ATP citrate lyase large subunit

Table 2.2. Descriptions and sequences of fungi obtained from GenBank used in the phylogenetic analyses

				GenBank accession numbers[ab]					
Isolate	**Species**	**Host**	**Origin**	**ITS**	**EF1-α**	**RPB1**	**RPB2[c]**	**cmdA**	**acl1**
NRRL20438	*Fusarium ambrosium*	*Euwallacea fornicatus*	India	AF178397	AF178332	JX171470	JX171584	...	...
NRRL22346	*F. ambrosium*	*E. fornicatus*	India	EU329669	FJ240350	KC691587	EU329503	...	...
NRRL62942	*F. ambrosium*	*Camellia sinensis*	Sri Lanka	KM406631	KM406624	KM406638	KM406645	...	...
NRRL54727	*F. euwallaceae*	*E. whifordiodendrus*	Israel	JQ038019	JQ038012	JQ038026	JQ038033	...	...
NRRL62626	*F. euwallaceae*	*Persea americana*	CA (USA)	KC691560	KU171722	KU171682	KU171702	...	...
UCR3641[c]	*F. kuroshium*	*Platanus racemosa*	CA (USA)	KX262196	KX262216	KX262236	KX262256	...	...
UCR3654	*F. kuroshium*	*P. americana*	CA (USA)	KX262201	KX262221	KX262241	KX262261	...	...
NRRL32434	*F. lichenicola*	*Homo sapien*	Germany	DQ094444	DQ246977	HM347156	EF470161	...	...
NRRL22468	*F. neocosmosporiellum*	*Arachis hypogaea*	Guinea	DQ094318	AF178349	KC691616	EU329512	...	...
NRRL43467	*F. neocosmosporiellum*	*H. sapien*	LA (USA)	EF453092	EF452940	HM347178	EF469979	...	...
NRRL62578	*F. oligoseptatum*	*E. validus*	PA (USA)	KC691565	KC691537	KC691595	KC691626,KC691655	...	...
NRRL62579	*F. oligoseptatum*	*E. validus*	PA (USA)	KC691566	KC691538	KC691596	KC691627,KC691656	...	...
UCR4674	*Fusarium* sp. [AF-13]	*Euwallacea sp.*	Taichung, Taiwan	KX262208	KX262228	KX262248	KX262268	...	...
UCR4675	*Fusarium* sp. [AF-13]	*Euwallacea sp.*	Taichung, Taiwan	KX262209	KX262229	KX262249	KX262269	...	...
UCR4672	*Fusarium* sp. [AF-14]	*Euwallacea sp.*	Taichung, Taiwan	KX262206	KX262226	KX262246	KX262266	...	...
UCR4681	*Fusarium* sp. [AF-14]	*Euwallacea sp.*	Taichung, Taiwan	KX262215	KX262235	KX262255	KX262275	...	...
UCR4679	*Fusarium* sp. [AF-15]	*Euwallacea sp.*	Taichung, Taiwan	KX262213	KX262233	KX262253	KX262273	...	...
UCR4673	*Fusarium* sp. [AF-16]	*Euwallacea sp.*	Taichung, Taiwan	KX262207	KX262227	KX262247	KX262267	...	...
UCR4676	*Fusarium* sp. [AF-16]	*Euwallacea sp.*	Taichung, Taiwan	KX262210	KX262230	KX262250	KX262270	...	...
UCR4677	*Fusarium* sp. [AF-16]	*Euwallacea sp.*	Taichung, Taiwan	KX262211	KX262231	KX262251	KX262271	...	...
UCR4678	*Fusarium* sp. [AF-16]	*Euwallacea sp.*	Taichung, Taiwan	KX262212	KX262232	KX262252	KX262272	...	...
UCR4680	*Fusarium* sp. [AF-16]	*Euwallacea sp.*	Taichung, Taiwan	KX262214	KX262234	KX262254	KX262274	...	...
NRRL62590	*Fusarium* sp. [AF-6]	*Euwallacea* sp.	FL (USA)	KC691574	KC691546	KC691604	KC691635,KC691664	...	...
NRRL62591	*Fusarium* sp. [AF-6]	*Euwallacea* sp.	FL (USA)	KC691573	KC691545	KC691603	KC691634,KC691663	...	...
NRRL66088	*Fusarium* sp.	*Delonix regia*	FL (USA)	KM406632	KM406632	KM406639	KM406646	...	...
NRRL62941	*Fusarium* sp. [AF-10]	unknown	Singapore	KM406633	KM406626	KM406640	KM406647	...	...
NRRL62944	*Fusarium* sp. [AF-11]	*C. sinensis*	Sri Lanka	KM406634	KM406627	KM406641	KM406648	...	...
NRRL62606	*Fusarium* sp. [AF-3]	*Euwallacea* sp.	FL (USA)	KC691561	KC691533	KC691591	KC691622,KC691651	...	...
NRRL62629	*Fusarium* sp. [AF-3]	*E. interjectus*	FL (USA)	KC691564	KC691536	KC691594	KC691625,KC691654	...	...
NRRL22231	*Fusarium* sp. [AF-5]	*Hevea brasiliensis*	Malaysia	KC691570	KC691542	KC691600	KC691631,KC691660	...	...
NRRL46518	*Fusarium* sp. [AF-5]	*H. brasiliensis*	Malaysia	KC691571	KC691543	KC691601	KC691632,KC691661	...	...
NRRL62610	*Fusarium* sp. [AF-7]	*Euwallacea* sp.	Australia	KC691575	KC691547	KC691605	KC691636,KC691665	...	...
NRRL62611	*Fusarium* sp. [AF-7]	*Euwallacea* sp.	Australia	KC691576	KC691548	KC691606	KC691637,KC691666	...	...
NRRL62584	*Fusarium* sp. [AF-8]	*Euwallacea* sp.	FL (USA)	KC691582	KC691554	KC691612	KC691643,KC691672	...	...
NRRL62585	*Fusarium* sp. [AF-8]	*Euwallacea* sp.	FL (USA)	KC691577	KC691549	KC691607	KC691638,KC691667	...	...
NRRL22643	*Fusarium* sp. [AF-9]	*Xyleborus ferrigineus*	Costa Rica	KC691583	DQ247628	KC691613	KC691644,KC691673	...	...
JCM 9300	*Graphum basitruncatum*	Forest soil	Salomon Islands	AB038427	KJ131248	...	...	...	...
CMW12418	*G. carbonarium*	*Salix babylonica*	China	FJ434980	HM630602	...	...	...	...
CMW12420[c]	*G. carbonarium*	*S. babylonica*	China	FJ434989	HM630603	...	...	...	...
UCR2300	*G. carbonarium*	*A. auriculiformis*	Vietnam	KM592370	KM592362	...	...	...	...
UCR2325	*G. carbonarium*	*R. communis*	Vietnam	KM592372	KM592364	...	...	...	...
UCR2329	*G. carbonarium*	*R. communis*	Vietnam	KM592373	KM592365	...	...	...	...
UCR2308	*G. euwallaceae*	*Acacia auriculiformis*	Vietnam	KM592371	KM592363	...	...	...	...
UCR2980[c]	*G. euwallaceae*	*Persea americana*	CA (USA)	KF540224	KF534805	...	...	...	...
UCR2981	*G. euwallaceae*	*P. americana*	CA (USA)	KF540225	KF534806	...	...	...	...
CMW30626[c]	*G. fabiforme*	*Adansonia rubrostipa*	Madagascar	GQ200616	HM630592	...	...	...	...
CMW30627	*G. fabiforme*	*A. rubrostipa*	Madagascar	GQ200617	HM630593	...	...	...	...
CMW5605[c]	*G. fimbriisporum*	*Picea abies*	France	AY148177	HM630590	...	...	...	...
CMW5606	*G. fimbriisporum*	*P. abies*	Austria	AY148180	HM630591	...	...	...	...
UCR4593[c]	*G. kuroshium*	*P. americana*	CA (USA)	KX262276	KX262286	...	...	...	...
UCR4594[d]	*G. kuroshium*	*P. americana*	CA (USA)	KX262277	KX262287	...	...	...	...
CMW5601[c]	*G. laricis*	*Larix decidua*	Austria	AY148183	HM630588	...	...	...	...
CMW5603	*G. laricis*	*L. decidua*	Austria	AY148182	HM630589	...	...	...	...
CMW5292	*G. penicillioides*	*Populus nigra*	Czech Republic	HQ335310	HM630600	...	...	...	...
CMW5295	*G. penicillioides*	*P. nigra*	Czech Republic	HQ335311	HM630601	...	...	...	...
CMW12285	*G. pseudormiticum*	*Tsuga dumosa*	China	HM630608	HM630587	...	...	...	...
CMW503c	*G. pseudormiticum*	*Pinus* sp.	South Africa	AY148186	HM630586	...	...	...	...
UCR2159	*Graphium* sp. I	*Ailanthus altissima*	PA (USA)	KJ131228	KJ131238	...	...	...	...
UCR2162	*Graphium* sp. I	*A. altissima*	PA (USA)	KJ131231	KJ131241	...	...	...	...
UCR2163	*Graphium* sp. I	*A. altissima*	PA (USA)	KJ131232	KJ131242	...	...	...	...
UCR2132	*Graphium* sp. II	*Durio* sp.	Thailand	KM592367	KM363259	...	...	...	...
UCR2137	*Graphium* sp. II	*Durio* sp.	Thailand	KJ131236	KJ131246	...	...	...	...
UCR2140	*Graphium* sp. II	*Durio* sp.	Thailand	KJ131237	KJ131247	...	...	...	...
UCR2289	*Graphium* sp. III	*A. auriculiformis*	Vietnam	KM592368	KM592360	...	...	...	...
UCR2291	*Graphium* sp. III	*A. auriculiformis*	Vietnam	KM592369	KM592361	...	...	...	...
CBS485.77	*P. inflatum*	*H. sapien*	India	KM231829	KP012649	...	KM232395	KM231415	KM231065
UCR2993	*P. pembeum*	*Euwallacea* sp.	CA (USA)	KP012602	KP012642	...	KT936353	KT936374	KT936332
UCR2995	*P. pembeum*	*Euwallacea* sp.	CA (USA)	KP012604	KP012644	...	KT936355	KT936376	KT936334
UCR2330	*Paracremonium* sp. I	*R. communis*	Vietnam	KP030841	KP030849	...	KT936338	KT936359	KT936317
UCR2359	*Paracremonium* sp. I	*R. communis*	Vietnam	KP030842	KP030850	...	KT936339	KT936360	KT936318
CBS137.35	*X. recifei*	*H. sapien*	Brazil	KM231833	KM231968	...	KM232397	KM231419	KM231069
CBS188.82	*X. recifei*	*H. sapien*	Netherlands	KP012607	KP012647	...	KT936340	KT936361	KT936319
CBS541.89	*X. recifei*	Forest soil	Brazil	KM231834	KM231969	...	KM232398	KM231420	KM231070
CBS220.84	*Xenoacremonium recifei*	*P. americana*	CA (USA)	KP012608	KP012648	...	KT936341	KT936362	KT936320
CBS125165	*Nectria cinnabarina*	*Aesculus* spp.	France	MH856245	HM484656	...	KM232402	KM231424	KM231074
CBS108944	*Bifusarium dimerum*	*H. sapien*	Netherlands	JQ434586	KR673912	...	KM232363	KM231365	KM230996

[a]ITS = internal transcribed spacer region; EF1-α = translation elongation factor 1-α

[b]RPB = DNA-directed RNA polymerase II subunit; cmdA = calmodulin; acl1 = ATP citrate lyase large subunit

[c]Two accession numbers correspond to un-joined RPB2-1 and RPB2-2 sequences

[d]Denotes type-specimens

Table 2.3. List of the mating type (MAT) oligonucleotides developed targeting AFC species

Oligo ID	Sequence (5'→ 3')	T_m (°C)	GC (%)
AFC-MAT1F	GGTACATTGCCGAGATCAG	56.6	52.6
AFC-MAT1R	TATCTCCCTGAGTATGGACCT	55.3	47.6
AFC-MAT2F	CAACGACSTTGTTGCAG	54.3	52.9
AFC-MAT2R	CCAGGATCTGAGCTAAAGAA	54.7	45

Table 2.4. Representative beetle species proportion and AFC mating type proportion from collection sites in Taiwan

	***Euwallacea fornicatus* species complex**			**Mating type**[a]	
Field Collection Date	**PSHB (%)**	**TSHB (%)**	**KSHB (%)**	***MAT1-1-1* (%)**	***MAT1-2-1* (%)**
2017 log batch 1	92.6	4.9	2.5	…	…
2017 log batch 2	85.5	14.5	…	54.5	45.5
2017 log batch 3	78.2	18.7	3.1	60	40
2017 field batch	95	5	…	…	…
2018 field batch	97.5	2.5	…	33.3	66.6

[a] MAT assay was not performed on 2017 log batch 1 and 2017 field batch since fungi were not linked to beetle vector type

Table 2.5. Results from novel mating type assays targeting AFC Fusaria from Taiwan and other invaded areas

					Mating type		
Isolate	**Species**	**Host**	**Location**	**AFC Designation**	***MAT1-1-1***	***MAT1-2-1***	**GenBank Accession**
NRRL62942	*F. ambrosium*	*Camellia sinensis*	Sri Lanka	AF-1	+	-	MK463687
NRRL62626	*F. euwallaceae*	*Persea americana*	California, USA	AF-2	+	-	MK463688
UCR4511	*F. euwallaceae*	*Platanus racemosa*	California, USA	AF-2	+	-	MK463681
MB12	*F. oligoseptatum*	*E. validus*	Pennsylvania, USA	AF-4	+	-	MK463715
MB14	*F. oligoseptatum*	*E. validus*	Pennsylvania, USA	AF-4	+	-	MK463712
NRRL 22231	*Fusarium* sp.	*Hevea brasiliensis*	Malaysia	AF-5	+	-	MK463686
KOD133	*Fusarium* sp.	*E. fornicatus*	Florida, USA	AF-6	+	-	MK463683
LL157	*Fusarium* sp.	*E. fornicatus*	Florida, USA	AF-6	-	+	MK463716
UCR6638	*Fusarium* sp.	*E. fornicatus*	Florida, USA	AF-6	+	-	MK463679
KOD1405	*Fusarium* sp.	*E. fornicatus*	Florida, USA	AF-8	-	+	MK463713
UCR6665	*Fusarium* sp.	*E. fornicatus*	Florida, USA	AF-8	-	+	MK463711
NRRL 66088	*Fusarium* sp.	*Delonix regia*	Florida, USA	AF-9	+	-	MK463685
NRRL 62941	*Fusarium* sp.	Unknown	Singapore	AF-10	+	-	MK463684
NRRL 62943	*Fusarium* sp.	*C. sinensis*	Sri Lanka	AF-11	-	+	MK463717
TW43	*F. kuroshium*	*P. americana*	Danei District, Taiwan	AF-12	+	-	MK463700
UCR6408	*F. kuroshium*	*Euwallacea* sp.	Danei District, Taiwan	AF-12	+	-	MK463692
UCR3641	*F. kuroshium*	*P. racemosa*	California, USA	AF-12	+	-	MK463689
UCR3645	*F. kuroshium*	*P. racemosa*	California, USA	AF-12	+	-	MK463680
UCR5584	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-13	-	+	…
UCR6394	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-13	-	+	…
UCR6403	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-13	-	+	…
UCR6409	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-13	-	+	MK463703
UCR6432	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-13	-	+	MK463705
TW2	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-14	-	+	…
TW56	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-14	+	-	MK463710
UCR5509	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-14	+	-	…
UCR5546	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-14	-	+	MK463702
UCR5499	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-14	-	+	…
UCR6436	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-14	-	+	MK463706
TW15	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-15	+	-	MK463695
TW45	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-15	-	+	MK463708
UCR6395	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-15	+	-	MK463690
TW1	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-16	+	-	MK463696
TW34	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-16	+	-	MK463697
TW37	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-16	+	-	MK463698
UCR5508	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-16	+	-	…
UCR5513	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-16	-	+	…
UCR6405	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-16	+	-	MK463691
TW40	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-17	+	-	MK463699
UCR5545	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-17	-	+	…
UCR6414	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-17	+	-	MK463693
TW44	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-18	-	+	MK463693
TW55	*Fusarium* sp.	*P. americana*	Danei District, Taiwan	AF-18	-	+	MK463709
UCR5557	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-18	+	-	…
UCR6411	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-18	-	+	MK463704
UCR6417	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-18	+	-	MK463694
AFH1	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-hybrid species	+	-	MK463682
KOD1406	*Fusarium* sp.	*Euwallacea* sp.	Danei District, Taiwan	AF-hybrid species	+	-	MK463714

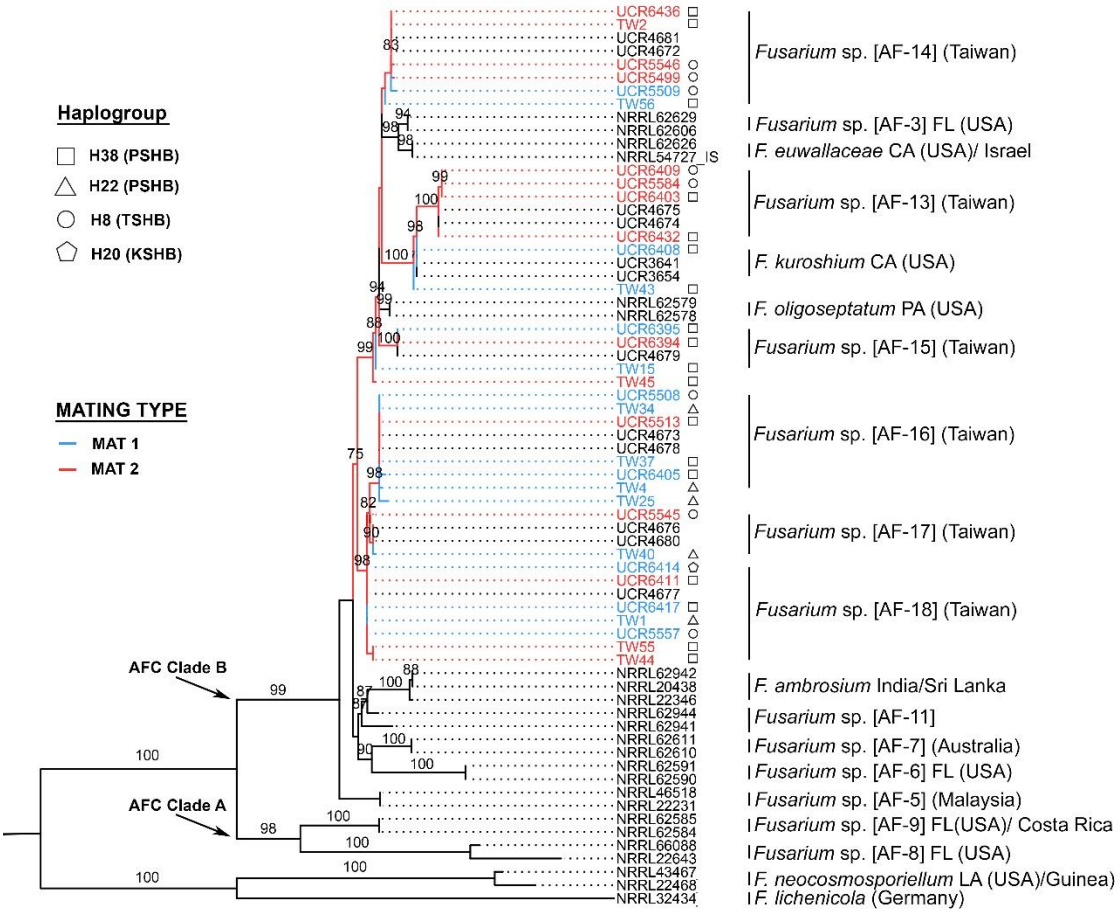

Figure 2.1. Multilocus phylogenetic analysis of AFC fusaria conducted with four genes: ribosomal internal transcribed spacer (ITS), elongation factor 1-α (EF1-α), DNA-directed RNA polymerase II largest subunit (RPB1), DNA-directed RNA polymerase II second largest subunit (RPB2). Diagram was constructed using IQ-TREE maximum likelihood method bootstrapped with 1000 replications. The black symbols to the right of the isolate indicate the beetle vector the fungi were recovered from while the blue or red color pertains to the *MAT* type determined from each isolate

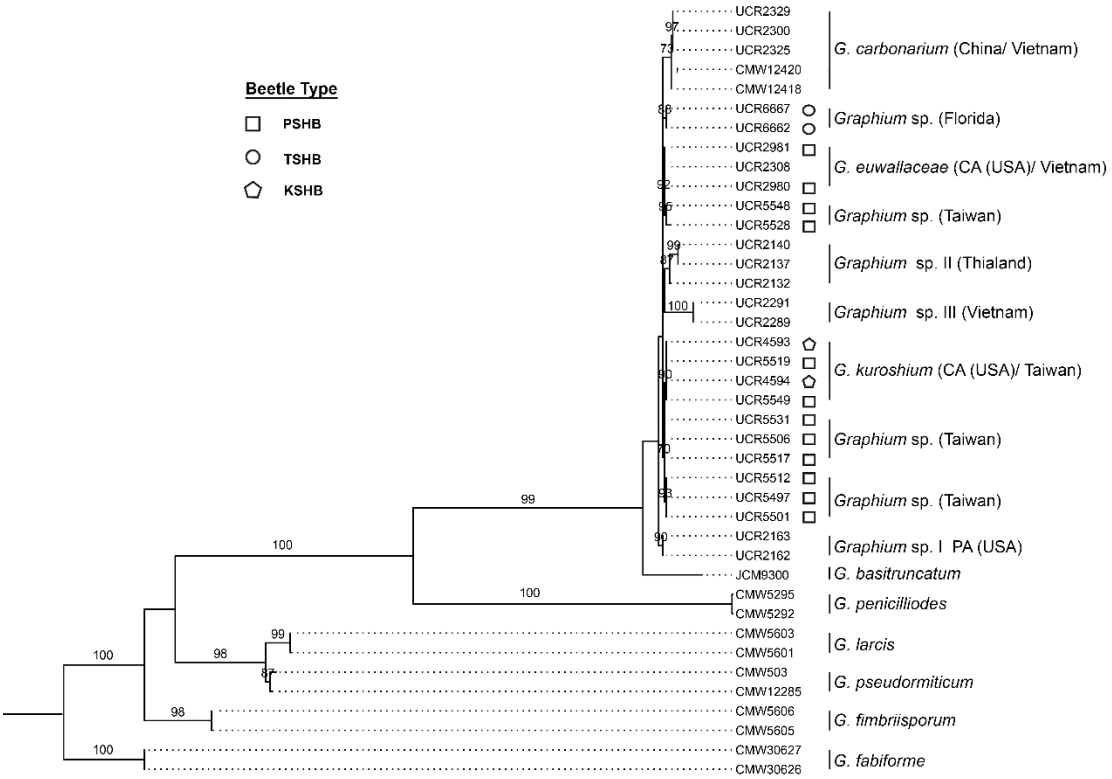

Figure 2.2. Multilocus phylogenetic analysis of *Graphium* spp. conducted with two genes: ribosomal internal transcribed spacer (ITS) and elongation factor 1-α (EF1-α). Diagram was constructed using IQ-TREE maximum likelihood method bootstrapped with 1000 replications. The black symbols to the right of the isolate indicate the beetle vector the fungi were recovered from.

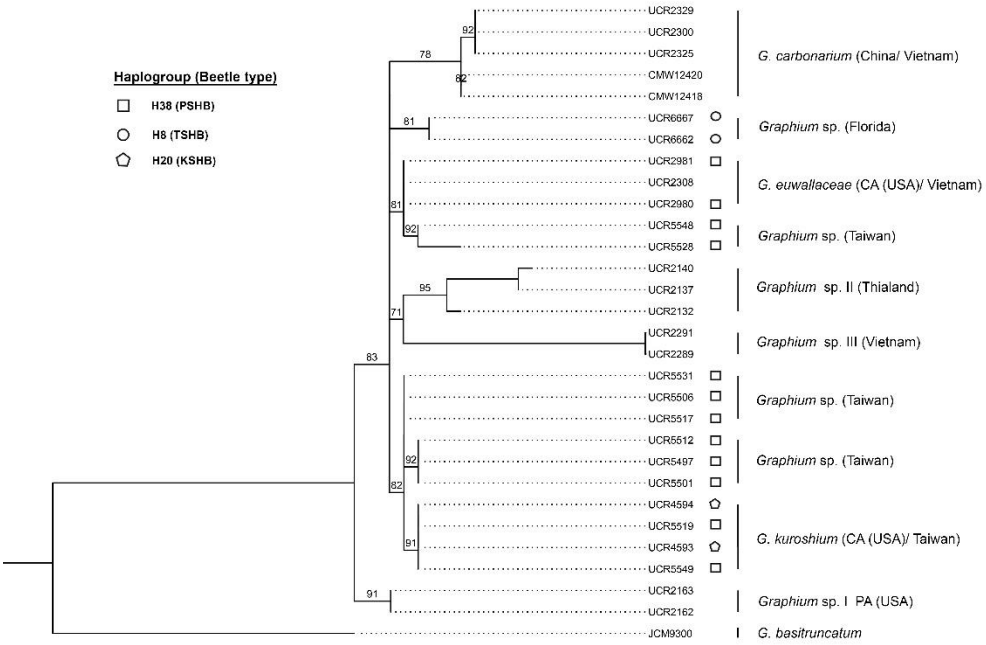

Figure 2.3. Multilocus phylogenetic analysis of *Graphium* spp. focused on *Grapihum* spp. associated with wood boring beetles conducted with two genes: ribosomal internal transcribed spacer (ITS) and elongation factor 1-α (EF1-α). Diagram was constructed using IQ-TREE maximum likelihood method bootstrapped with 1000 replications. The black symbols to the right of the isolate indicate the beetle vector the fungi were recovered from.

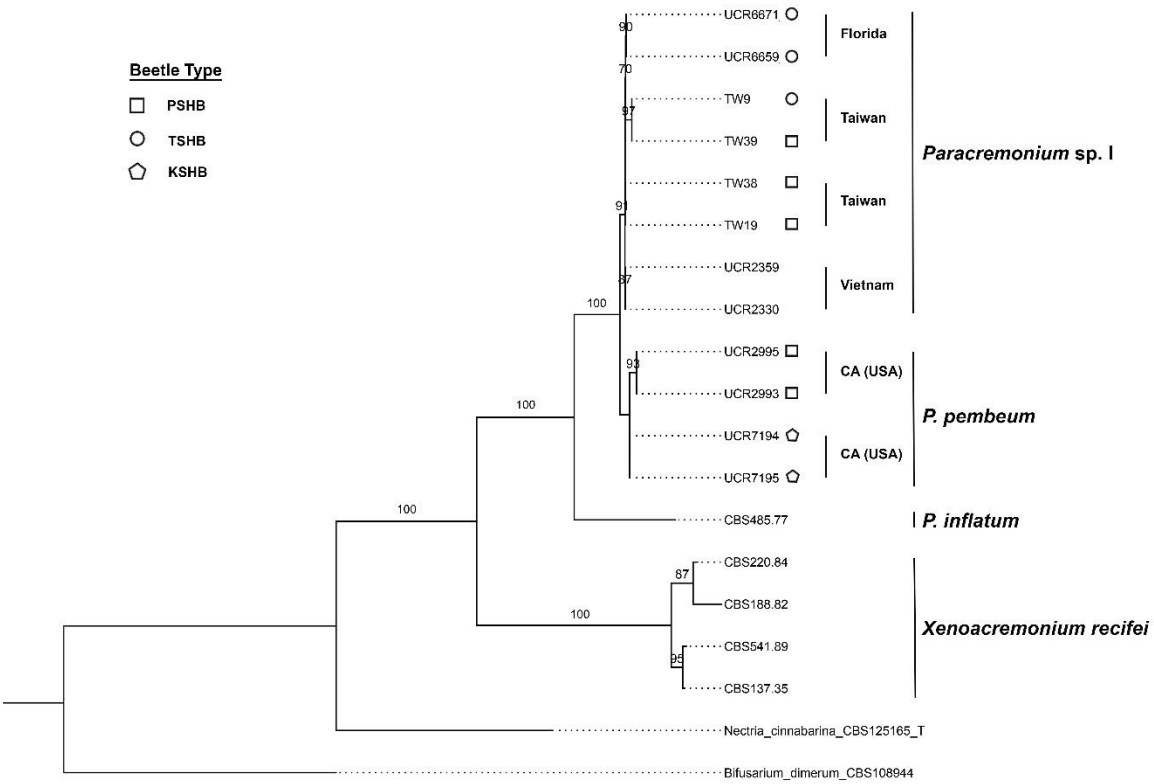

Figure 2.4. Multilocus phylogenetic analysis of *Paracremonium* spp. conducted with five genes: ribosomal internal transcribed spacer (ITS) and elongation factor 1-α (EF1-α), DNA-directed RNA polymerase II second largest subunit (RPB2), Calmodulin region (cmdA), and ATP citrate lyase region (alc1). Diagram was constructed using IQ-TREE maximum likelihood method bootstrapped with 1000 replications. The black symbols to the right of the isolate indicate the beetle vector the fungi were recovered from.

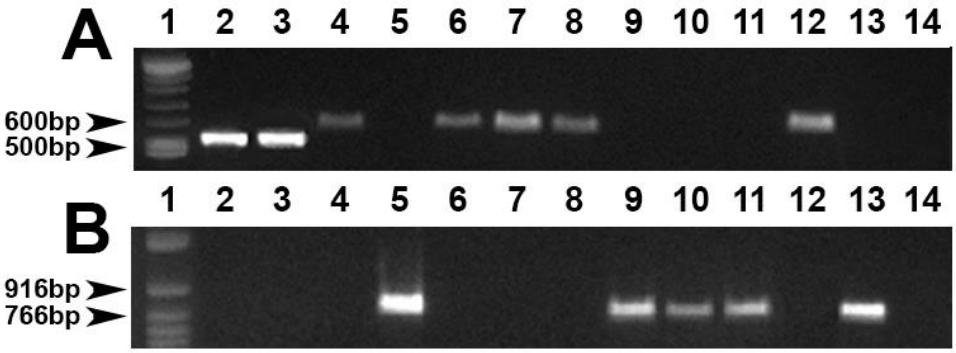

Figure 2.5. Agarose gel image of products from the AFC *MAT* type PCR assay. (A) shows products from heterothallic isolates containing the *MAT1-1-1* gene while (B) shows products from isolates containing *MAT1-2-1* gene. Lanes 2-13 represent AF-2, AF-12, AF-16, AF-13, AF-13, AF-15, AF-17, AF-14, AF-13, AF-18, AF-6, AF-8, respectively. Lane 14 is a non-treated control.

CHAPTER III. Fungal symbionts of the invasive ambrosia beetles *Euwallacea whitfordiodendrus* and *E. kuroshio* can support both beetle systems on artificial media

ABSTRACT

Ambrosia beetles (Coleoptera: Curculionidae) in the subfamily Scolytinae represent one of the most diverse and evolutionarily successful clades of insects, due in part to their mutualistic symbioses with fungi. These relationships are fairly specific, such that different ambrosia beetle species will only associate with certain fungi. The extent of this specificity is unknown for the vast majority of ambrosia beetle-fungal complexes, yet has important implications for the success of these beetles and fungi in their surrounding environments. The ambrosia beetles *Euwallacea whitfordiodendrus* and *E. kuroshio* are invasive in California, and together with their phytopathogenic ambrosia fungi are responsible for the emerging plant disease Fusarium Dieback, which can reproduce in over sixty local tree species. The two beetle species exist sympatrically in some areas of their invasive range, raising the additional concern that they may be able to exchange symbionts or that their symbionts may hybridize to produce a strain with increased virulence or pathogenicity. Here we perform symbiont switching experiments on artificial media, to determine if *E. whitfordiodendrus* and *E. kuroshio* can survive and reproduce on each other's symbiotic fungi. We found reproduction is maximized on the beetles' primary *Fusarium* fungal symbionts, regardless of which *Euwallacea* and *Fusarium* species were paired, indicating that there is potential for symbiont switching in natural populations of these beetles. From our experiments, the putative roles of the primary

versus auxiliary fungal symbionts, as well as the implications of these roles for beetle reproduction and development in their surrounding environment, are discussed.

INTRODUCTION

Ambrosia beetles belonging to the subfamily Scolytinae within weevils (Coleoptera: Curculionidae) are *predominantly* xylomycetophagous and rely on mutualistic relationships with fungi to complete their life cycle in the trunks and branches of trees (Beaver 1989). Their ability to successfully farm these "ambrosia" fungi inside of trees has made them a concern when introduced into non-native habitats, where some species have been reported to cause considerable economic damage to local flora (Cognato et al. 2015; Hanula et al. 2008; Hulcr and Stelinski 2017; O'Donnell et al. 2016), as well as agriculturally important commodities (Danthanarayana 1968; Mendel et al. 2012; Eskalen et al. 2013). The evolution of specialized glandular organs called mycangia (Francke-Grosmann 1956; Batra 1963) enables the beetles to vector nutritious fungal propagules from host to host, facilitating a mutualistic symbiosis between both partners. Mechanical injury to wood throughout the boring process enables rapid penetration of the fungal propagules and initiation of fungal gardens, which the beetles feed upon (Batra 1985). The fungal symbionts have historically been thought to have species-specific associations with ambrosia fungi (Batra 1963), but recent evidence suggests that these insect-fungus interactions can be promiscuous among some mutualisms (Carrillo et al. 2014; Kostovcik et al. 2015).

Ambrosia beetles in the genus *Euwallacea* are thought to be native to Southeast Asia (Rabaglia et al. 2006) and are considered pests of economic importance in many

areas they have invaded. One of the earliest described cases was that of *E. fornicatus* Eichoff (1868), commonly known as the tea shot hole borer (TSHB), with its fungal mutualist *Fusarium ambrosium* (Gadd and Loos). This beetle-fungus complex was reported in India and Sri Lanka, where it has been a significant pest of tea for over a century (Gadd 1941; Danthanarayana 1968). In California (Eskalen et al. 2013), Israel (Mendel et al. 2012), and South Africa (Paap et al. 2018), *E. whitfordiodendrus* Schedl (1942), commonly known as the polyphagous shot hole borer (PSHB), was reported to cause the disease Fusarium Dieback (FD) through the spread of its symbiont *F. euwallaceae* (S. Freeman, Z. Mendel, T. Aoki & O'Donnell), in addition to the fungi *Graphium euwallaceae* (M. Twizeyimana, S.C. Lynch & A. Eskalen), and *Paracremonium pembeum* (S.C. Lynch & Eskalen), within susceptible hosts (Eskalen et al. 2013; Lynch et al. 2016). In 2014, another closely related species, *E. kuroshio* Gomez & Hulcr (2018), known commonly as the Kuroshio shot hole borer (KSHB), was also detected in California and found to cause FD by association with *Fusarium kuroshium* (F. Na, J. D. Carrillo & A. Eskalen), as well as *Graphium kuroshium* (F. Na, J. D. Carrillo & A. Eskalen) (Na et al. 2018). *E. fornicatus*, *E. whitfordiodendrus*, and *E. kuroshio* are all members of the cryptic *Euwallacea fornicatus* species complex (Cooperband et al. 2016; Stouthamer et al. 2017). Recent studies of *Euwallacea* spp. adult female mycangia from invaded areas recovered mainly *Fusarium* spp. from the *F. solani* species complex (FSSC) clade 3 in a monophyletic clade termed the ambrosia fusaria clade (AFC) (Kasson et al. 2013; O'Donnell et al. 2015). The dominance of AFC members within female mycangia suggest they are the primary symbionts the beetles associate with.

The invasive range of *E. whitfordiodendrus* and *E. kuroshio* in California overlaps in some areas, including Orange and San Diego Counties (Chapter I). These pests and the fungi they carry are of great concern for land managers and avocado growers in California due to their ability to colonize a wide range of host tree species. Currently, both can colonize, reproduce in, and cause severe damage to 15 different hosts that are common in the California landscape (Eskalen 2018; Freeman et al. 2013b; Lynch et al. 2016; Na et al. 2018). It is currently unknown if these beetles invaded California with fungal symbionts that are exclusive to each species, or if there is any affinity of the beetles towards mutualism with an alternate fungal complex. Events of fungal sharing or promiscuity have been reported in other ambrosia beetle systems (Hulcr and Cognato 2010; Kostovcik et al. 2015; Skelton et al. 2019; Carrillo et al. 2014; Saucedo-Carabez et al. 2018). Opportunities exist for *E. whitfordiodendrus* and *E. kuroshio* to encounter each other's fungal symbionts in their invasive range, but their ability to survive and reproduce on an alternate but closely related fungal complex, and the effects of this switch, has not been investigated to date.

Obligate specificity of *E. whitfordiodendrus* to *F. euwallaceae* was previously investigated; it was found that larvae placed on petri dishes colonized with *F. ambrosium* (associated with *E. fornicatus*), *F. mangiferae,* and *F. oxysporum* all had significantly higher death rates than when fed *F. euwallaceae* (Freeman et al. 2013a). However, phylogenetic analysis from a later study showed that a *Euwallacea* sp. recovered from Florida (likely invasive *E. fornicatus*) was associated with two different AFC members: *Fusarium* sp. [AF-6] from AFC clade B and *Fusarium* sp. [AF-8] from AFC clade A

(O'Donnell et al. 2015). Early work done in this field revealed that ambrosia beetles may not feed solely on a primary symbiont throughout its life cycle, but may also feed on auxiliary fungi (Batra 1967). Thus, their diet likely consists of the entire microbial complex present in the fungal garden, including bacteria and other fungi, although the proportions of various fungi and bacteria consumed can vary with the life stage of the beetle, age of the gallery, and host tree species (Batra 1966; Biedermann et al. 2013; Haanstad and Norris 1985). Artificial diet studies help to reveal the function and importance of such auxiliary fungi on ambrosia beetle survival and reproduction. In *E. whitfordiodendrus*, Freeman et al. (2015) showed that *F. euwallaceae* and *G. euwallaceae* are suitable food sources for larvae, while *P. pembeum* alone was not sufficient for larval development.

The objectives of this study were to (i) investigate fungal symbiont fidelity of aseptic virgin *E. whitfordiodendrus* and *E. kuroshio* on both "natural" (their own) and "alternate" (their congener's) diets of *F. euwallaceae, G. euwallaceae, P. pembeum, F. kuroshium*, and *G. kuroshium* given in individual and combination treatments; (ii) evaluate the occurrence and proportion of each symbiont in different life stages of the beetles; and (iii) compare offspring counts of *E. whitfordiodendrus* and *E. kuroshio* across all treatments as a measure of fitness. We also (iv) investigate the ability of both mated and aseptic virgin *E. whitfordiodendrus* to survive on non-symbiotic *F. solani* and *F. oxysporum* recovered from avocado wood in California by counting offspring and recovered fungal colony forming units (CFU) from female mycangia.

METHODS AND MATERIALS

Beetle collection and rearing. *E. whitfordiodendrus* (PSHB) and *E. kuroshio* (KSHB) were collected in 2014 from infested avocado groves (*Persea americana* Mill cv. "Hass") in Los Angeles and San Diego Counties, respectively. The beetles were collected live from funnel traps baited with quercivorol (Dodge et al. 2017) or from brood galleries by dissecting wood from infested host trees, and confirmed as PSHB and KSHB through quantitative polymerase chain reaction (qPCR) analysis as described by Rugman-Jones and Stouthamer (2016). Beetles were surface disinfested with 70% ethanol for 10 s and dried on filter paper then introduced into artificial sawdust-based media (75 g avocado sawdust, 20 g agar, 10 g sucrose, 5 g casein, 5 g starch, 5 g yeast, 1 g Wesson's salt mixture, 2.5 ml wheat germ oil, and 5 ml 95% ethanol in 500 ml of distilled water) modified from Peer and Taborsky (2004). Sawdust was generated from avocado logs >10 cm in diameter, collected from Hass cultivars from uninfested groves in Agricultural Operations at UC Riverside; wood was collected using a chop saw and stored at -20ºC until ready for use. The collected beetles were reared in Insectary and Quarantine Facility at the University of California, Riverside (UCR) and subsequent generations were used for fungal diet studies. Prepared media was autoclaved, thoroughly mixed, and poured hot, to reduce viscosity, into 50 ml centrifuge tubes (Falcon brand) up to the 25 ml mark. Tubes were allowed to dry in a sterile laminar flow hood to evaporate condensed moisture, and then capped until use. Before introduction of beetles to the tubes, holes were poked ~2mm deep into the media, using a sterilized 2mm diameter metal rod, to facilitate boring activity. After beetles were introduced, the caps were left loose to allow

gas exchange and kept at a constant temperature and relative humidity of 24°C and ~40%, respectively.

Preparation of aseptic virgin *E. whitfordiodendrus* and *E. kuroshio.* It has been shown that ambrosia beetles are generally free of their fungal symbiont when eclosion occurs outside of their natal galleries (Batra 1966). To obtain aseptic virgin beetles, 20-30 inseminated female beetles of both species were introduced into individual sawdust media tubes and allowed to establish galleries for 21-25 days, or until numerous pupae were visible in some of the tubes. Tubes were dissected, and viable F1 female pupae from each beetle species were removed using a fine miniature paint brush cleaned with 95% ethanol and allowed to dry before handling pupae. The pupae were placed in clean glass petri dishes lined with sterile Kimwipes® moistened with sterile distilled water. The dishes were wrapped with Parafilm® (Neenah, WI, USA) to prevent desiccation of pupae, and replenished with sterile distilled water as needed. No more than 15 pupae were added to each dish, to prevent overcrowding and potential loss from mycosis. Pupae eclosed after 1-7 days, and teneral virgin females were surface sterilized with 70% ethanol and used for downstream diet experiments. A general survey was conducted to quantify symbiotic fungi from aseptic virgin females versus non-virgin females to verify mycangia were free of symbionts and the results are presented in Table 1.

Fungal symbiont fidelity of *E. whitfordiodendrus* and *E. kuroshio.* Fungal symbionts used in this study were *F. euwallaceae*, (NRRL 62626), *G. euwallaceae* (CBS 140035), and *P. pembeum* (CBS 140042) previously acquired from *E. whitfordiodendrus* (Freeman et al. 2013b; Lynch et al. 2016), as well as *F. kuroshium* (CBS142642) and *G. kuroshium*

(CBS142643) previously acquired from *E. kuroshio* (Na et al., 2018). The plant pathogen *Lasiodiplodia citricola* (Botryosphaeriaceae; UCR6961), recovered from an avocado branch canker near shot hole borer galleries in San Diego County, was used as a negative control. To prepare the fungal treatments, spore suspensions from isolates of *F. euwallaceae, F. kuroshium, G. euwallaceae, G. kuroshium,* and *P. pembeum* were made by scraping one-week old sporulating cultures with a sterile inoculating loop and streaking onto fresh potato dextrose agar (PDA; BD Difco, Sparks, MD, USA) media plates amended with 0.01% tetracycline hydrochloride (PDA-t). Plates were grown at 25°C for three days, after which 5 ml of sterile distilled water was added to each plate and agitated with sterile bent glass rods to release the spores into suspension. The suspension was passed through a layer of sterile Kimwipe® to remove large mycelial fragments. All spore suspensions were quantified using a Bright-Line hemocytometer (Hausser Scientific, Horsham, PA, USA) and diluted to $1x10^6$ spores/ml. Sawdust media tubes were inoculated by depositing a total of 10 µl of spore suspension onto the surface of the media and spread with a sterile glass pestle to distribute spores evenly across the surface. Fungal spore suspensions of beetle symbionts were confirmed as the desired species thorough real-time qPCR using species-specific primers and probes described in Chapter I. Fungal plugs (3mm) of 1-week old *L. citricola* were taken with a cork borer and placed mycelium side down on the media surface in lieu of a spore suspension. The fungal diet treatments were as follows: *F. euwallaceae, G. euwallaceae, F. euwallaceae + G. euwallaceae* (5 µl of each suspension), *P. pembeum, F. kuroshium, G. kuroshium, F. kuroshium + G. kuroshium* (5 µl of each suspension), *L. citricola* (negative control),

and a non-treated control (NTC) consisting of an uninoculated media tube. After fungal inoculation, the diet tubes were incubated at 25°C for three days before introducing the aseptic virgin female beetles (one per tube). The experiment was designed using a randomized complete block design with five subsamples spread across five trials, with each trial representing a block. Within each block, the nine treatments were applied to the two different beetle species at random and replicated five times for a total of 45 observations per beetle species per trial. Beetles in the genus *Euwallacea* undergo arrhenotokous parthenogenesis (Kirkendall et al. 2015); thus a virgin female will produce only male F1 offspring, which she can then mate with to produce F2 daughters. After ~70 days, when the F2 generation females were present, the diet tubes were dissected and all life stages (eggs, larvae, pupae, and adults) were counted. Pupae and adults were sexed to record sex ratio, and larvae, pupae, and adults were sampled (2 of each from each colony tube when possible) to investigate fungal fidelity in different sexes and life stages. Offspring were quickly euthanized in 70% ethanol, and immediately processed to recover the fungi.

Isolation and quantification of fungal symbionts from offspring. Beetle offspring obtained from artificial diet tubes were surface sterilized by submersion in 70% ethanol and vortexed for 5 s, rinsed with sterile deionized water, and allowed to dry on sterile filter paper. All samples were processed individually except larvae, which were pooled into one sample (two larvae per sample). Additionally, adult female beetle heads were separated from the thoracic and abdominal segments under a dissection microscope and processed separately to investigate differences between the female head (which contains

the mycangia) and female body (guts). All samples were macerated in 1.5 ml microcentrifuge tubes with sterile plastic pestles. The macerated samples were suspended in 1 ml of sterile 0.01% triton (except for adult female bodies, which were suspended in 200 µl) and further diluted at different rates depending on the sample type: 1:1 dilution for female heads, 3:1 dilution of entire males, larvae, and pupae, and no dilution for female bodies. 25 µl of suspension from each sample was pipetted onto three replicate petri dishes containing PDA-t and spread using sterile glass L-shaped rods. Plates were incubated for two days at 25°C. One of the three plates was used to acquire 5 single-spore fungal colonies, sampled at random, and a plug was transferred to a plate containing ½ strength PDA, mycelium side down. After one week of incubation at 25°C the resultant colonies were grouped by morphology (Freeman et al. 2013b; Lynch et al. 2016; Na et al. 2018) and representatives of each group of interest were identified using qPCR with species-specific probes and primers targeting the symbiont species of interest (Chapter I). Information was recorded using custom-built forms on the iForm application (Zurion Software Inc., Herndon, VA, USA). Common contaminant data, such as *Penicillium* spp., *Aspergillus* spp., and *Rhizopus* spp., was not recorded. The remaining PDA plates were allowed to grow for one week at 25°C and total colony forming units (CFU) were counted. *Fusarium* spp., *Graphium* spp., and *P. pembeum* were distinguished based on previous descriptions of morphology on PDA (Freeman et al. 2013b; Lynch et al. 2016; Na et al. 2018).

Aseptic virgin and non-virgin female *E. whitfordiodendrus* survival on non-symbiotic *Fusarium* spp. Fungal symbionts used in this experiment were *F. euwallaceae*, (NRRL 62626), *F. solani* (UCR7265, UCR7266 recovered from *Platanus racemosa* (Orange County) and avocado (San Diego County) wood, respectively) and *F. oxysporum* (UCR3424, UCR6846 recovered from avocado wood from Orange and Riverside Counties, respectively). To prepare the fungal treatments, spore suspensions were prepared from isolates following the methods described above to a final concentration of 1x 10^6 spores/ml, and media tubes were inoculated with 10 µl of each species. The fungal diet treatments were as follows: *F. euwallaceae, F. solani, F. oxysporum,* and a non-treated control (NTC). Experimental design was a randomized complete block design with sub-sampling and five trials representing the blocks. Within each block, the six treatments were applied to two groups (aseptic virgin and non-virgin females) and replicated five times for a total of 30 observations per beetle status per trial. Aseptic virgin females were incubated for 80 days to observe females in the F2 generation (F1 is all male) while non-virgin females were incubated for 40 days to observe females in the F1 generation. Individual eggs, larvae, pupae, and adult male and female offspring were counted with two individuals sampled from each group when possible. All recovered individuals were sampled, surface sterilized, and processed as mentioned above with an additional sampling of the gallery wall with a sterile wooden toothpick. This was done by opening the media to expose the inner gallery wall, rotating the tip of the toothpick 360° in the gallery, and suspending the wooden toothpick in 1 ml sterile 0.01% triton. The beetle samples were diluted to the ratios reported in previous

sections, and gallery samples were not diluted. 25 µl of suspension from each sample was pipetted onto three petri dishes containing PDA-t and spread using sterile glass L-shaped rods. Plates were incubated for one week at 25°C and total colony forming units (CFU) were counted. *F. euwallaceae, F. solani, and F. oxysporum* were distinguishing using previous descriptions of morphology (Freeman et al., 2013b, Leslie and Summerell, 2006). In this experiment, recovery of *Fusarium* spp. from the head of the mycangia was compared to recovery from the gallery and female body.

Statistical analyses. Statistical analyses were performed using R v3.4.3 (Team 2017) or Stata (StataCorp 2017). Offspring count data was found to be overdispersed, as the variance exceeds the mean, using the package "COUNT" (Hilbe 2016) in R. A negative binomial regression was used to analyze the data using the "MASS" package (Venables & Ripley 2002) in R. Analysis of Variance (ANOVA) was used to compare independent variables in the regression model using the *anova* function in R. Pairwise comparisons between *E. whitfordiodendrus* and *E. kuroshio* were performed by sample type within each treatment, as well as across all treatments for total offspring counts using estimated marginal means with the "emmeans" package (Lenth et al. 2018) in R. For CFU spore count data was transformed by using the square root of the counts then, Poisson regression analysis using a generalized linear model was performed using the "MASS" package in R. Post-hoc analysis for CFU data was done by constructing a contrast matrix in order to compare all responses to fungal treatments between *E. whitfordiodendrus* and *E. kuroshio* by sample type, then by using the "multcomp" package (Hothorn et al. 2008) in R with link function *glht* for comparison of recovered CFU counts between *E.*

whitfordiodendrus and *E. kuroshio* based on the matrix. For analysis of specific fungal species recovered from randomly sampled spores within *E. whitfordiodendrus* and *E. kuroshio* offspring across all treatments, a multinomial logistic regression analysis was performed in STATA using the "mlogit" syntax (StataCorp 2017), comparing recovery of fungi from all life stages within each fungal treatment. P-values for multinomial logistic regression were generated using Wald tests for each independent variable. All bar plots were generated using "ggplot2" (Wickham et al. 2016) in R. Negative control (*L. citricola*) and NTC data were only considered in total offspring analysis.

RESULTS

***E. whitfordiodendrus* and *E. kuroshio* fecundity on artificial diets.** The number of offspring of each life stage (egg, larva, pupa, and adult) was compared between beetle and fungal treatments. Total offspring counts reflect the sum of all life stages, to give the total number of offspring within a colony tube. ANOVA revealed significant differences in offspring counts (for all life stages, as well as total) between fungal treatments ($P < 0.05$) but not between beetle species ($P = 0.469$). Estimated marginal means (EMM) analysis of the negative binomial regression model revealed significant differences ($P < 0.05$) between fungal treatment groups (Fig. 3.1). All treatment groups and combination groups that included either *F. euwallaceae* and *F. kuroshium*, considered the primary symbionts of *E. whitfordiodendrus* and *E. kuroshio,* respectively, had higher offspring counts compared to treatments without the primary symbionts (Fig. 1). Treatments with auxiliary fungi *G. euwallaceae*, *G. kuroshium,* and *P. pembeum* had offspring counts significantly greater ($P < 0.05$) than the negative control (*L. citricola*) and the NTC (Fig.

3.1). Offspring counts were broken down into life stage groups with five levels (adult females, adult males, pupae, larvae, and eggs). Direct comparisons between *E. whitfordiodendrus* and *E. kuroshio* across all treatments showed significant difference ($P < 0.05$) only between egg counts when treated with *F. euwallaceae* + *G. euwallaceae*, adult males when treated with *F. kuroshium* only, and adult females when treated with *G. euwallaceae* (Fig. 3.2). Difference in number of males between PSHB and KSHB; PSHB produced more ($P < 0.001$)

Fungal species recovery within *E. whitfordiodendrus* and *E. kuroshio* life stages. The proportion of fungal species recovered from different beetle species within each fungal treatment shows consumption, primarily of the fungi provided in the artificial diet (Table 3.4; Fig. 3.4). Within each treatment, multinomial logistic regression analysis compared *E. whitfordiodendrus* and *E. kuroshio* (Table 3.2). Within the *F. euwallaceae* treatment, recovery of *P. pembeum* was significantly greater through analysis of regression coefficients in *E. whitfordiodendrus* than in *E. kuroshio* ($P < 0.05$). This was also found in the *F. kuroshium* + *G. kuroshium* treatment, where *F. kuroshium* recovery was greater in *E. whitfordiodendrus* than in *E. kuroshium* ($P < 0.05$). Different life stages were also compared within each treatment and beetle species (Table 3.3). Within the *F. euwallaceae* fungal treatment, recovery of *Graphium* spp. and *P. pembeum* from larvae was significantly higher when compared to adult female bodies ($P < 0.05$), while *P. pembeum* was recovered significantly more ($P < 0.05$) in males compared to adult female bodies. However, *G. euwallaceae* was not recovered from any female head within the *F. euwallaceae* treatment from random spore sampling. When fed *F. euwallaceae* and *G.*

euwallaceae in combination, female heads, larvae, and males had significantly less *G. euwallaceae* compared to recovery from the female bodies, while *P. pembeum* recovery was significantly less in males than female bodies ($P < 0.05$). Within the *F. kuroshium* treatment, *P. pembeum* was recovered significantly more from larvae than female bodies ($P < 0.05$). Within a combination treatment of *F. kuroshium* and *G. kuroshium*, recovery of *F. kuroshium* was significantly greater in female heads compared to the female bodies sampled, while being recovered significantly less in larvae compared to female bodies ($P < 0.05$).

Quantification of fungal genera within *E. whitfordiodendrus* and *E. kuroshio* life stages. Recovery of fungal genera (*Fusarium* spp., *Graphium* spp., and *P. pembeum*.) revealed significant differences ($P<0.05$) in EMM from Poisson regression models when contrasting by sample types. General trends when removing the treatment variable from the model showed CFUs recovered from *Fusarium* spp. had the highest EMM in female heads compared to all other sample types including female bodies, males, and larvae. While *Graphium* spp. were recovered from larvae with the highest EMM compared to other sample types, *P. pembeum* was also recovered from larvae with the highest EMM compared to other groups (Figs 3.3.1-3.3.4). In-depth analyses into fungal recovery within different sample types based on fungal treatment are visualized in Figures 3-6. Treatments that contained *F. euwallaceae* or *F. kuroshium* individually or in combination with *Graphium* spp. resulted in significantly greater ($P <0.05$) *Fusarium* spp. allocated in the female head compared to other fungal genera recovered (Fig. 3.3.1). In treatments without primary symbionts *F. euwallaceae* or *F. kuroshium*, the female heads contained

significantly higher CFU counts of the auxiliary fungi they were treated with (Fig. 3.3.1). Generally, CFUs recovered from female bodies, males, and larvae were composed of more auxiliary fungal genera, specifically *Graphium* spp., even in the presence of the primary symbionts (Figs. 3.3.2-3.3.4). For example, in female bodies in both *E. whitfordiodendrus* and *E. kuroshio* given a treatment of *F. euwallaceae* + *G. euwallaceae* or *F. kuroshium* + *G. kuroshium,* recovery of *Graphium spp.* was significantly higher ($P < 0.05$) than recovery of *Fusarium* spp. or *P. pembeum* (Fig. 3.3.2) This trend was also seen in larvae and males, except in males when *E. kuroshium* was fed *F. euwallaceae* + *G. euwallaceae* (Figs. 3.3.3-3.3.4). Overall, the fungal genera recovered from both *E. whitfordiodendrus* and *E. kuroshio* across all treatments were predicted by fungal treatment, providing evidence that all life stages were consuming fungi provided in the artificial diet treatments (Table 3.4; Figs 3.3.1-3.3.4; 3.4).

Aseptic virgin and non-virgin *E. whitfordiodendrus* fecundity on non-symbiotic *Fusarium* spp. Total offspring analysis of counts by treatment from the negative binomial regression model from non-virgin females fed non-symbiotic *Fusarium* spp. showed significant differences ($P < 0.05$) in the NTC and the primary symbiont (*F. euwallaceae*) treatment compared to the non-symbiotic *F. solani* and *F. oxysporum* treatments based on EMM (Fig. 3.5.1). The primary symbiont treatment *F. euwallaceae* produced the most offspring compared to all other treatments provided. For aseptic virgin females, fewer offspring were observed overall than in non-virgin females (Fig 3.5.2). The NTC and *F. oxysporum* treatments were the least productive, while *F. solani* produced significantly more offspring than the former according to EMM. As was seen

with the non-virgin females, the primary symbiont treatment of *F. euwallaceae* produced the most total offspring.

Quantification of *Fusarium spp.* within *E. whitfordiodendrus* female mycangia. Colony forming unit counts of *F. euwallaceae* from non-virgin female heads (mycangia) had the highest EMM across all treatments, but the EMM was highest in the primary symbiont (*F. euwallaceae*) and NTC treatment (Fig. 3.6). Non-symbiotic fungi *F. solani* and *F. oxysporum* were recovered from female heads when beetles were treated with the respective fungal species, but the EMM were significantly less ($P < 0.05$) than that of *F. euwallaceae* in female mycangia. Analysis of virgin female heads showed similar trends as the non-virgin trials with *F. euwallaceae* recovered consistently across all treatments except *F. oxysporum.* The non-symbiont treatment of *F. oxysporum* yielded neither *F. euwallaceae* nor the former in the mycangia, although both were present in the galleries (Fig. 3.6). When aseptic virgins were treated with non-symbiont *F. solani*, the recovery of this fungus was higher than the primary symbiont *F. euwallaceae* but not significant ($P > 0.05$) according to EMM. Recovered fungi from other sampled individuals (female bodies, larvae, males) and gallery samples in both non-virgin and aseptic virgin trials confirmed that *E. whitfordiodendrus* were consuming the fungi provided, whether symbiotic or non-symbiotic (Fig. 3.6).

DISCUSSION

Investigation into the fungal symbiont fidelity of two invasive ambrosia beetles *E. whitfordiodendrus* and *E. kuroshio* through artificial diet experiments indicate that both species can survive on each other's symbiotic ambrosia fungi. Diets including the

primary AFC symbionts *F. euwallaceae* and *F. kuroshium* produced the most total offspring, with no significant differences between *E. whitfordiodendrus* and *E. kuroshio* when reared on either fungus. Symbiosis of *E. whitfordiodendrus* with *F. euwallaceae* was previously reported to be specific and exclusive (Freeman et al. 2013a); however, the present study provides evidence for a more promiscuous symbiosis. This along with other studies (Kasson et al. 2013; Mendel et al. 2012; Na et al. 2018; O'Donnell et al. 2015) showed that the AFC Fusaria are primary symbionts and are necessary for optimal fecundity of both *E. whitfordiodendrus* and *E. kuroshio*. When supplied as a sole food source, the auxiliary fungi *G. euwallaceae*, *G. kuroshium*, and *P. pembeum* led to reduced fecundity but could all support at least one cycle of metamorphosis. We recovered offspring from one *E. kuroshio* female on the negative control treatment *L. citricola*, but upon further analysis found that all fungi isolated from these offspring were *G. kuroshium*, indicating that downstream contamination occurred and that these offspring were feeding upon a fungal symbiont rather than our negative control.

The removal of female pupae from brood galleries such that they eclose in an aseptic environment has been reported for diet experiments previously (Batra 1966; Baker and Norris 1968), and is advantageous when performing in vitro switching experiments because it frees the mycangia of fungal symbionts. This makes it possible to introduce specific fungi to callow females with empty mycangia in order to study effects on fecundity, fitness, and fungal uptake and consumption when reared on alternative fungi, as shown in the present study. When introducing aseptic virgin *E. whitfordiodendrus* and *E. kuroshio* females to various fungi that were initially thought to

be exclusive to their respective beetle vectors, similar numbers of total offspring were produced regardless of whether the fungi were previously associated with that beetle species or not (Figs. 3.1-3.2). The high fecundity from treatments that include *F. euwallaceae* or *F. kuroshium* indicate they are a significant food source that positively contributes to offspring production from females, which corroborates previous studies that AFC Fusaria are the primary symbionts of *Euwallacea* spp. (Kasson et al. 2013; O'Donnell et al. 2015). The addition of auxiliary fungi *G. euwallaceae* and *G. kuroshium* to treatments including AFC Fusaria was not found to significantly increase fecundity, while auxiliary fungal treatments alone produced substantially fewer offspring (Figs 1-2), similar to observations on the ambrosia beetle *Xyloborus ferrugineus* that appears to be associated with *Graphium* spp. (Baker and Norris 1968). Auxiliary fungal treatments alone often produced only one generation of males, while treatments including AFC fusaria in the diet produced a second generation with females over the duration of the experiment. The auxiliary fungi may not be suitable for optimal female longevity and offspring production but are shown here to support a cycle of metamorphosis in both *E. whitfordiodendrus* and *E. kuroshio.* As these pests can attack and reproduce in living trees (Cooperband et al. 2016; Eskalen et al. 2013), host tree species may select for one symbiont over another because of active host defenses (Freeman et al. 2015). This makes it advantageous for *Euwallacea* spp. to possess a suite of symbionts, increasing chances of survival and offspring production on both primary symbionts, like AFC Fusaria, and auxiliary symbionts, such as *Graphium* spp. and *P. pembeum*, when necessary.

Graphium euwallaceae was previously found in significant quantities (CFU) in female bodies as well as larvae of *E. whitfordiodendrus* and is suggested to be the dominant food source of immature beetle stages (Freeman et al. 2015). In this study, the proportion (Table 3.4; Fig. 3.4) and quantity (Figs. 3.3.1-3.3.4) of *Graphium* spp. in the female body, larvae, and males were generally higher than what was found in female mycangia in both beetle species. However, the proportions and quantities were largely dependent on the fungal symbiont(s) the individual aseptic virgin foundresses were initially treated with. For example, *Graphium* spp. recovery was substantially higher from female bodies, males, and larvae when added in combination with AFC Fusaria compared to the AFC Fusaria treated alone (Fig 3.3.2-3.3.4). Similarly, when treated with auxiliary *G. euwallaceae, G. kuroshium,* or *P. pembeum* alone, greater amounts of each respective auxiliary fungus were recovered from all sample types including female foundress mycangia (Table 3.4; Figs 3.3.1-3.3.4; 3.4). This is significant for *E. kuroshio* since it has not been previously described to be associated with *P. pembeum*, but more so because auxiliary fungi are generally not dominant in female mycangia; rather, the primary symbiont AFC Fusaria is usually dominant in *Euwallacea* spp. (Kasson et al. 2013). This indicates that entry of the spores into mycangium happens passively as the aseptic virgin foundresses are exposed to the fungi in the galleries, as was suggested by early work on ambrosia beetle mycangia (Batra 1967). In a diet tube inoculated with the negative control *L. citricola*, which was not expected to enter the female mycangia, mycelial propagules from a single foundress were recovered from dilution plating the female head, but no offspring were produced (data not shown). Conversely, in *E. whitfordiodendrus*

non-virgin diet experiments inoculated with non-symbiotic fungi *F. solani* and *F. oxysporum*, the primary symbiont *F. euwallaceae* dominated the female mycangia even with evidence of the non-symbiont in the galleries and guts of individuals (Fig. 3.6). This may be explained by the previous occupation of *F. euwallaceae* in the inseminated non-virgin foundresses mycangia, but does not explain how emerging female offspring are able to select for the primary symbiont AFC Fusaria over the non-symbiont *Fusarium* spp. present in the galleries (Fig. 3.6). *P. pembeum* has been recently proposed to possibly serve as a regulator of contaminants in galleries (Freeman et al. 2015), similar to antimicrobial actinomycetes like *Streptomyces* spp. in attine ants (Mueller and Gerardo 2002) and may contribute to this phenomenon, but should be further investigated to see if *P. pembeum* has a beneficial function in selective loading of *Euwallacea* spp. mycangia.

F. solani and *F. oxysporum* are well known plant pathogens reported in many host species, mainly as soil-borne pathogens (Coleman 2016; Ploetz 2006) but also as endophytes in some plant species (Evans et al. 2003; Promputtha et al. 2007). The isolates used in this study were not confirmed pathogenic strains, but were used to explore the effects of non-symbiotic *Fusarium* spp. on offspring production and the potential of these ambrosia fungi to load *E. whitfordiodendrus* female mycangia and replace *F. euwallaceae*. The ability of non-virgin foundresses to survive and produce offspring was suppressed by the presence of non-symbiont *Fusarium* spp. treatments compared to *F. euwallaceae* and NTC treatments (Fig. 3.5.1). The difference in affinity of *F. solani* compared to *F. oxysporum*, particularly in aseptic virgin female experiments (Fig. 3.6), was also interesting since *F. solani* is more closely related to *F. euwallaceae*,

both being from the FSSC. Generally, females retained more symbiotic *F. euwallaceae* than non-symbiotic *Fusarium* spp. in their mycangia (Fig. 3.6), which may explain their survival even with pressure from a non-symbiotic fungus. The release of symbiont fungal propagules is thought to occur during gallery formation by the females (Batra 1966; Beaver 1989). In this study, the non-virgin foundresses may have been able to establish some of the primary symbiont AFC Fusaria while also moving the non-symbiotic *Fusarium* spp. deeper into uncolonized parts of the media. In conclusion, we discovered it is possible for *E. whitfordiodendrus* females to survive and produce offspring under heavy pressure of non-symbiotic *Fusarium* spp. through the selective retainment of their primary AFC symbiont in female mycangia.

In previous studies (Batra 1966; Baker and Norris 1968), as well as the present study, it proved difficult to completely disassociate ambrosia beetles from their fungal symbionts (Table 3.1). Although the virgin mycangia were verified free of symbionts using our isolation methods, there were still individuals where symbiotic fungi, mainly the auxiliary fungi *Graphium* spp. or *P. pembeum*, persisted in the body of the virgin female foundresses post-eclosion and were recovered from offspring. For instance, several female head samples (two of *E. whitfordiodendrus*, one of *E. kuroshio*), we recovered both *F. euwallaceae* and *F. kuroshium*. This indicates that the mandibular mycangia of species within the *Euwallacea fornicatus* species complex are capable of simultaneously housing multiple species of primary symbiont, in addition to auxiliary fungi and bacteria. Similarily, *E. whitfordiodendrus* female bodies fed an alternate diet of *G. kuroshium* retained *G. euwallaceae,* and likewise *E. kuroshio* that were fed *G.*

euwallaceae retained *G. kuroshium* (Table 3.4; Fig. 3.4). Selective media has been used to study primary symbionts such as *Raffalea lauricola* associated with *Xyloborus glabratus* using cycloheximide (Fraedrich et al. 2007). Similar approaches could be developed to control diet environments and study effects of the primary and auxiliary fungi on *Euwallacea* spp. as well as other ambrosia beetle systems in diet experiments.

The presence of *E. whitfordiodendrus* and *E. kuroshio* in California is already a great concern for land managers, arborists, and homeowners because of the pests' polyphagous nature and wide host range. The potential of symbiont sharing adds additional concern for invaded areas where both exist sympatrically. The transmission of fungi into female mycangia is at least partially dependent on the content of the gallery. Inoculation of the xylem often extends past the point of beetle-spread inoculation and can invade nearby galleries in the same host, providing opportunities for horizontal transmission between beetles in close proximity (Carrillo et al. 2014; Hulcr and Cognato 2010). This a major concern because all symbionts associated with *E. whitfordiodendrus* and *E. kuroshio* have been described as plant pathogens (Eskalen et al. 2012; Freeman et al. 2013b; Lynch et al. 2016; Na et al. 2018) with varying degrees of virulence (Na et al. 2018). Recombination in plant pathogenic fungi has been widely studied (Millgroom 1996; Orr-Weaver and Szostak 1985) and can facilitate new genetic combinations, hybridization, and horizontal gene transfer, which can cause clonal emergence of new phytopathogenic lineages (Fisher et al. 2012). Although ambrosia fungi are thought to generally forego sexual reproduction (Beaver 1989), sexual stages have been observed in *Raffaelea vaginata* recovered from ambrosia beetles in South Africa (Musvuugwa et al.

2015), as well as in the mycangial fungus *Ambrosiella cleistominuta*, associated with the ambrosia beetle *Anisandrus maiche* (Mayers et al. 2017). *Fusarium* spp. are known to recombine sexually (Leslie & Summerell 2006) as well as parasexually (Correll et al. 1987), and this may also be a possibility in the AFC Fusaria associated with *E. whitfordiodendrus* and *E. kuroshio* if interactions between their symbionts occur in nature.

The ability of *E. whitfordiodendrus* and *E. kuroshio* to survive on alternate diets has significant implications for California, where both species exist sympatrically, and no practical approach to managing them exists. Currently, strategies to control the pests are limited (Eatough Jones et al. 2017; Eatough Jones and Paine 2015; Mayorquin et al. 2018), due to their cryptic nature inside woody hosts and the difficulty of delivering effective treatments. Tree removal is recommended in severe cases (Eatough Jones and Paine 2015). These findings highlight a need for more research geared toward understanding insect-microbe interactions in ambrosia beetle systems, to uncover ways to disrupt this system and control spread in invaded areas.

LITERATURE CITED

Baker, J.M. and Norris, D.M., 1968. A complex of fungi mutualistically involved in the nutrition of the ambrosia beetle Xyleborus ferrugineus. Journal of Invertebrate Pathology, 11(2), 246-250.

Batra, L.R., 1963. Ecology of ambrosia fungi and their dissemination by beetles. Transactions of the Kansas Academy of Science (1903-), 66(2), 213-236.

Batra, L.R., 1966. Ambrosia fungi: extent of specificity to ambrosia beetles. Science, 153(3732), 193-195.

Batra, L.R., 1967. Ambrosia fungi: a taxonomic revision, and nutritional studies of some species. Mycologia, 59(6), 976-1017.

Batra, L.R., 1985. Ambrosia beetles and their associated fungi: research trends and techniques. Proceedings: Plant Sciences, 94(2-3), 137-148.

Beaver RA. 1989. Insect–fungus relationships in the bark and ambrosia beetles. In: Wilding N, Collins NM, Hammond PM, Webber JF (eds). Insect–fungus Interactions. Academic Press: London, UK, 121–143.

Biedermann, P. H., Klepzig, K. D., Taborsky, M. & Six, D. L. 2013. Abundance and dynamics of filamentous fungi in the complex ambrosia gardens of the primitively eusocial beetle Xyleborinus saxesenii Ratzeburg (Coleoptera: Curculionidae, Scolytinae). FEMS Microbiol Ecol*,* 83**,** 711-23.

Carrillo, D., Duncan, R.E., Ploetz, J.N., Campbell, A.F., Ploetz, R.C. and Peña, J.E., 2014. Lateral transfer of a phytopathogenic symbiont among native and exotic ambrosia beetles. Plant Pathol., 63(1), 54-62.

Cognato, A.I., Hoebeke, E.R., Kajimura, H. and Smith, S.M. 2015. History of the exotic ambrosia beetles Euwallacea interjectus and Euwallacea validus (Coleoptera: Curculionidae: Xyleborini) in the United States. Journal of economic entomology, 108(3), 1129-1135.

Coleman, J.J., 2016. The Fusarium solani species complex: ubiquitous pathogens of agricultural importance. Molecular plant pathology, 17(2), 146-158.

Correll, J.C., Klittich, C.J.R. and Leslie, J.F., 1987. Nitrate nonutilizing mutants of Fusarium oxysporum and their use in vegetative compatibility tests. Phytopathology, 77(12), 1640-1646.

Danthanarayana, W., 1968. The distribution and host-range of the shot-hole borer (*Xyleborus fornicatus* Eich.) of tea. Tea Quarterly. 39, 61–69.

Dodge, C., Coolidge, J., Cooperband, M., Cossé, A., Carrillo, D. & Stouthamer, R. 2017. Quercivorol as a lure for the polyphagous and Kuroshio shot hole borers, *Euwallacea* spp. nr. *fornicatus* (Coleoptera: Scolytinae), vectors of Fusarium dieback. PeerJ, 5**,** e3656.

Eatough Jones, M., Kabashima, J., Eskalen, A., Dimson, M., Mayorquin, J.S., Carrillo, J.D., Hanlon, C.C. and Paine, T.D., 2017. Evaluations of insecticides and fungicides for reducing attack rates of a new invasive ambrosia beetle (Euwallacea sp., Coleoptera: Curculionidae: Scolytinae) in infested landscape trees in California. Journal of economic entomology, 110(4), 1611-1618.

Eatough Jones, M. & Paine, T. D. 2015. Effect of Chipping and Solarization on Emergence and Boring Activity of a Recently Introduced Ambrosia Beetle (Euwallacea sp., Coleoptera: Curculionidae: Scolytinae) in Southern California. J Econ Entomol*,* 108**,** 1852-9.

Eskalen, A. 2018. *Host Species* [Online]. Available: https://ucanr.edu/sites/pshb/overview/SHB_Reproductive_Hosts/ [Accessed].

Eskalen, A., Gonzalez, A., Wang, D. H., Twizeyimana, M., Mayorquin, J. S. & Lynch, S. C. 2012. First Report of a *Fusarium* sp. and Its Vector Tea Shot Hole Borer (*Euwallacea fornicatus*) Causing Fusarium Dieback on Avocado in California. Plant Dis*.,* 96**,** 1070-1070.

Eskalen, A., Stouthamer, R., Lynch, S. C., Rugman-Jones, P. F., Twizeyimana, M., Gonzalez, A., & Thibault, T. 2013. Host Range of Fusarium Dieback and Its Ambrosia Beetle (Coleoptera: Scolytinae) Vector in Southern California. Plant Dis., 97(7), 938–951.

Evans, H.C., Holmes, K.A. and Thomas, S.E., 2003. Endophytes and mycoparasites associated with an indigenous forest tree, Theobroma gileri, in Ecuador and a preliminary assessment of their potential as biocontrol agents of cocoa diseases. Mycological Progress, 2(2), 149-160.

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L. and Gurr, S.J., 2012. Emerging fungal threats to animal, plant and ecosystem health. Nature, 484(7393), 186.

Fraedrich, S.W., Harrington, T.C., Rabaglia, R.J., Ulyshen, M.D., Mayfield Iii, A.E., Hanula, J.L., Eickwort, J.M. and Miller, D.R., 2008. A fungal symbiont of the redbay ambrosia beetle causes a lethal wilt in redbay and other Lauraceae in the southeastern United States. Plant Disease, 92(2), 215-224.

Franke-Grosmann, H. 1956. Hautdrüsen als Träger der Pilzsymbiose bei Ambrosiakäfern. Z. Morph. u. Ökol. Tiere, 45, 275-308

Freeman, S., Sharon, M., Maymon, M., Mendel, Z., Protasov, A., Aoki, T., Eskalen, A., O'Donnell, K. 2013a. *Fusarium euwallaceae* sp. nov.--a symbiotic fungus of *Euwallacea sp*., an invasive ambrosia beetle in Israel and California. Mycologia, 105(6), 1595–1606.

Freeman, S., Protasov, A., Sharon, M., Mohotti, K., Eliyahu, M., Okon-Levy, N., Maymon, M., Mendel, Z. 2013b. Obligate feed requirement of *Fusarium sp*. nov., an avocado wilting agent, by the ambrosia beetle *Euwallacea* aff. *fornicata*. Symbiosis, 58(1-3), 245–251.

Freeman, S., Sharon, M., Dori-Bachash, M., Maymon, M., Belausov, E., Maoz, Y., Margalit, O., Protasov, A. and Mendel, Z., 2016. Symbiotic association of three fungal species throughout the life cycle of the ambrosia beetle Euwallacea nr. fornicatus. Symbiosis, 68(1-3), 115-128.

Gadd, C. H. & Loos, C. A. 1946. The ambrosia fungus of *Xyleborus fornicatus* Eich. Transactions British Mycological Society, 31, 13-19.

Gomez, D.F., Skelton, J., Steininger, M.S., Stouthamer, R., Rugman-Jones, P., Sittichaya, W., Rabaglia, R.J. and Hulcr, J., 2018. Species Delineation Within the Euwallacea fornicatus (Coleoptera: Curculionidae) Complex Revealed by Morphometric and Phylogenetic Analyses. Insect Systematics and Diversity, 2(6), 2.

Gaad, C. H. 1941. Observations on an attack by shot-hole borer on tea. The Tea Quarterly, 14, 132-146.

Gaad, C. H. & Loos, C. A. 1946. The ambrosia fungus of *Xyleborus fornicatus* Eich. Transactions British Mycological Society, 31, 13-19

Haanstad, J.O. and Norris, D.M., 1985. Microbial symbiotes of the ambrosia beetle Xyloterinus politus. Microbial Ecology, 11(3), 267-276.

Hanula, J.L., Mayfield III, A.E., Fraedrich, S.W. and Rabaglia, R.J., 2008. Biology and host associations of redbay ambrosia beetle (Coleoptera: Curculionidae: Scolytinae), exotic vector of laurel wilt killing redbay trees in the southeastern United States. Journal of Economic Entomology, 101(4), 1276-1286.

Hilbe, J. M. 2016. Count. *Functions,* Data and Code for Count Data*.* 1.3.4 ed.

Hothorn, T., Bretz, F. and Westfall, P., 2008. Simultaneous inference in general parametric models. Biometrical journal, 50(3), 346-363.

Hulcr, J., & Cognato, A. I. 2010. Repeated evolution of crop theft in fungus-farming ambrosia beetles. Evolution, 64(11), 3205–3212.

Hulcr, J., & Stelinski, L. L. 2016. The Ambrosia Symbiosis: From Evolutionary Ecology to Practical Management. Annu. Rev. Entomol., 62, 285-303.

Kasson, M. T., O'Donnell, K., Rooney, A. P., Sink, S., Ploetz, R. C., Ploetz, J. N., Konkol, J. L., Carrillo, D., Freeman, S., Mendel, Z., Smith, J. A., Black, A. W., Hulcr, J., Bateman, C., Stefkova, K., Campbell, P. R., Geering, A. D. W., Dann, E. K., Eskalen, A., Mohotti, K., Short, D. P. G., Aoki, T., Fenstermacher, K. A., Davis, D. D., Geiser, D. M. 2013. An inordinate fondness for *Fusarium*: Phylogenetic diversity of fusaria cultivated by ambrosia beetles in the genus *Euwallacea* on avocado and other plant hosts. Fungal Genet. Biol., 56, 147–157.

Kirkendall, L. R., Biedermann, P. H. W. & Jordal, B. H. 2015. Evolution and Diversity of Bark and Ambrosia Beetles. 85-156.

Kostovcik, M., Bateman, C. C., Kolarik, M., Stelinski, L. L., Jordal, B. H., & Hulcr, J. 2015. The ambrosia symbiosis is specific in some species and promiscuous in others: evidence from community pyrosequencing. ISME J., 9(1), 126–138

Lenth, R., Love, J. and Hervé, M., 2017. Package 'emmeans'. Underst Stat, 34, 216-21.

Leslie, J. F., & Summerell, B. A. 2008. The Fusarium laboratory manual. John Wiley & Sons.

Lynch, S. C., Twizeyimana, M., Mayorquin, J. S., Wang, D. H., Na, F., Kayim, M., ... & Hulcr, J. 2016. Identification, pathogenicity and abundance of *Paracremonium pembeum* sp. nov. and *Graphium euwallaceae* sp. nov.—two newly discovered mycangial associates of the polyphagous shot hole borer (*Euwallacea sp*.) in California. Mycologia, 108(2), 313-329.

Mayers, C. G., Harrington, T. C. & Ranger, C. M. 2017. First report of a sexual state in an ambrosia fungus: Ambrosiella cleistominuta sp. nov. associated with the ambrosia beetle Anisandrus maiche. Botany*,* 95**,** 503-512.

Mayorquin, J.S., Carrillo, J.D., Twizeyimana, M., Peacock, B.B., Sugino, K.Y., Na, F., Wang, D.H., Kabashima, J.N. and Eskalen, A. 2018. Chemical Management of Invasive Shot Hole Borer and Fusarium Dieback in California Sycamore (*Platanus racemosa*) in Southern California. Plant Dis., 102(7), 1307-1315.

Mendel, Z., Protasov, A., Sharon, M., Zveibil, A., Yehuda, S. Ben, O'Donnell, K., Rabaglia, R., Wysoki, M., Freeman, S. 2012. An Asian ambrosia beetle *Euwallacea fornicatus* and its novel symbiotic fungus *Fusarium sp.* pose a serious threat to the Israeli avocado industry. Phytoparasitica, 40(3), 235–238.

Milgroom, M.G., 1996. Recombination and the multilocus structure of fungal populations. Annual review of phytopathology, 34(1), 457-477.

Mueller, U.G. and Gerardo, N., 2002. Fungus-farming insects: multiple origins and diverse evolutionary histories. Proceedings of the National Academy of Sciences, 99(24), 15247-15249.

Musvuugwa, T., De Beer, Z.W., Duong, T.A., Dreyer, L.L., Oberlander, K.C. and Roets, F., 2015. New species of Ophiostomatales from Scolytinae and Platypodinae beetles in the Cape Floristic Region, including the discovery of the sexual state of Raffaelea. Antonie van Leeuwenhoek, 108(4), 933-950

.

Na, F., Carrillo, J.D., Mayorquin, J.S., Ndinga-Muniania, C., Stajich, J.E., Stouthamer, R., Huang, Y.T., Lin, Y.T., Chen, C.Y. and Eskalen, A., 2018. Two novel fungal symbionts Fusarium kuroshium sp. nov. and Graphium kuroshium sp. nov. of Kuroshio shot hole borer (Euwallacea sp. nr. fornicatus) cause Fusarium dieback on woody host species in California. Plant Dis., 102(6), 1154-1164.

O'Donnell, K., Sink, S., Libeskind-Hadas, R., Hulcr, J., Kasson, M. T., Ploetz, R. C., Konkol, J. L., Ploetz, J. N., Carrillo, D., Campbell, A., Duncan, R. E., Liyanage, P. N., Eskalen, A., Na, F., Geiser, D. M., Bateman, C., Freeman, S., Mendel, Z., Sharon, M., Aoki, T., Crossé, A. A., Rooney, A. P. 2015. Discordant phylogenies suggest repeated host shifts in the *Fusarium-Euwallacea* ambrosia beetle mutualism. Fungal Genet. Biol., 82, 277–290.

O'Donnell, K., Libeskind-Hadas, R., Hulcr, J., Bateman, C., Kasson, M.T., Ploetz, R.C., Konkol, J.L., Ploetz, J.N., Carrillo, D., Campbell, A. and Duncan, R.E., 2016. Invasive Asian Fusarium–Euwallacea ambrosia beetle mutualists pose a serious threat to forests, urban landscapes and the avocado industry. Phytoparasitica, 44(4), 435-442.

Orr-Weaver, T.L. and Szostak, J.W., 1985. Fungal recombination. Microbiological reviews, 49(1), 33.

Paap, T., De Beer, Z.W., Migliorini, D., Nel, W.J. and Wingfield, M.J., 2018. The polyphagous shot hole borer (PSHB) and its fungal symbiont Fusarium euwallaceae: a new invasion in South Africa. Australas. Plant Path., 47(2), 231-237.

Ploetz, R.C., 2006. Fusarium wilt of banana is caused by several pathogens referred to as Fusarium oxysporum f. sp. cubense. Phytopathology, 96(6), 653-656.

Promputtha, I., Lumyong, S., Dhanasekaran, V., McKenzie, E.H.C., Hyde, K.D. and Jeewon, R., 2007. A phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. Microbial Ecology, 53(4), 579-590.

Rabaglia, R.J., Dole, S.A. and Cognato, A.I., 2006. Review of American Xyleborina (Coleoptera: Curculionidae: Scolytinae) occurring north of Mexico, with an illustrated key. Annals of the Entomological Society of America, 99(6), 1034-1056.

Rugman-Jones, P.F. and Stouthamer, R., 2017. High-resolution melt analysis without DNA extraction affords rapid genotype resolution and species identification. Mol. Ecol. Resour., 17(4), 598-607.

Skelton, J., Johnson, A. J., Jusino, M. A., Bateman, C., Hulcr, J., 2019 A selective fungal transport organ (mycangium) maintains coarse phylogenetic congruence between fungus-farming ambrosia beetles and their symbionts. Proceedings of the Royal Society B

StataCorp. 2017. *Stata Statistical Software: Release 15*. College Station, TX: StataCorp LLC

Stouthamer, R., Rugman-Jones, P., Thu, P.Q., Eskalen, A., Thibault, T., Hulcr, J., Wang, L.J., Jordal, B.H., Chen, C.Y., Cooperband, M. and Lin, C.S., 2017. Tracing the origin of a cryptic invader: phylogeography of the E uwallacea fornicatus (C oleoptera: C urculionidae: S colytinae) species complex. Agricultural and forest entomology, 19(4), 366-375.

Team, R.C., 2013. R: A language and environment for statistical computing.

Venables, W.N. and Ripley, B.D., 2002. Tree-based methods. In Modern Applied Statistics with S (251-269). Springer, New York, NY.

Wickham, H., 2016. ggplot2: elegant graphics for data analysis. Springer.

Table 3.1. Aseptic virgin and non-virgin CFU content from artificial media

	Recovered Fungi (Mean CFU)		
Sample Type (*N*=35)[a]	*F. euwallaceae*	*G. euwallaceae*	*P. pembeum*
Virgin Head	-	-	-
Virgin Body	1.48	3.2	0.46
Non-Virgin Head	3252.57	17.14	0
Non-Virgin Body	1.26	5.71	0.46

[a] All individuals were derived from rearing tubes with sawdust media

Table 3.2. Multinomial logistic regression coefficients from each recovered fungal species based on the fungal treatment and beetle species

Fungal treatment[a]	**Beetle species**	**Recovered fungi from all individuals**				
		Coefficient (%)[bcd]				
		F. euwallaceae	*G. euwallaceae*	*P. pembeum*	*F. kuroshium*	*G. kuroshium*
Fusarium euwallaceae	*Euwallacea kuroshio*	-	-	-	-	-
	E.whitfordiodendrus	-	18.49	**1.34**	-18.11	-18.46
Graphium euwallaceae	*E. kuroshio*	-	-	-	-	-
	E.whitfordiodendrus	16.57	-	16.6	N/A	-17.52
F. euwallaceae +G. euwallaceae	*E. kuroshio*	-	-	-	-	-
	E.whitfordiodendrus	-	0.31	**-1.17**	-15.98	-15.52
Paracremonium pembeum	*E. kuroshio*	-	-	-	-	-
	E.whitfordiodendrus	17.98	17.24	-	-18.22	N/A
F. kuroshium	*E. kuroshio*	-	-	-	-	-
	E.whitfordiodendrus	N/A	17.19	0.54	-	-16.76
G. kuroshium	*E. kuroshio*	-	-	-	-	-
	E.whitfordiodendrus	*17.29*	17.38	-0.71	-16.56	-
F. kuroshium + G. kuroshium	*E. kuroshio*	-	-	-	-	-
	E.whitfordiodendrus	N/A	15.22	-0.4	**0.57**	-

[a] Fungal treatment analysis done individually

[b] "-" indicates the reference for each multinomial model conducted for each treatment

[c] "N/A" represents cases where a particular species of fungi was not recovered from any group

[d] Bold numbers indicate Wald-test significance at less than α=0.05

Table 3.3. Multinomial logistic regression coefficients from each recovered fungal species based on the beetle life stage and fungal treatment

		Recovered Fungi from Individuals				
Fungal treatment[a]	**Sample Type**	**Coefficient (%)[bcd]**				
		F. euwallaceae	*G. euwallaceae*	*P. pembeum*	*F. kuroshium*	*G. kuroshium*
Fusarium euwallaceae	*Female Bodies*	-	-	-	-	-
	Female Heads	-	-17.15	-18.62	-19.63	**-1.972**
	Larvae	-	**3.69**	**1.93**	-23.13	**2.5**
	Males	-	1.74	**1.23**	-0.87	0.31
Graphium euwallaceae	*Female Bodies*	-	-	-	-	-
	Female Heads	17.36	-	-1.08	N/A	-17.18
	Larvae	-0.516	-	-17.25	N/A	-1.59
	Males	15.29	-	-0.57	N/A	-1.91
F. euwallaceae + G. euwallaceae	*Female Bodies*	-	-	-	-	-
	Female Heads	-	**-3.72**	-18.83	-16.84	-0.07
	Larvae	-	**-2.61**	1.13	-13.75	3.01
	Males	-	**-1.04**	**-2.61**	0.12	17.04
Paracremonium Pembeum	*Female Bodies*	-	-	-	-	-
	Female Heads	*0.34*	-0.18	-	17.65	N/A
	Larvae	*-18.34*	18.21	-	0.672	N/A
	Males	*-2.02*	17.71	-	16.47	N/A
F. kuroshium	*Female Bodies*	-	-	-	-	-
	Female Heads	N/A	0.48	**-3.67**	-	13.31
	Larvae	N/A	-5.4	**2.52**	-	-5.45
	Males	N/A	18.17	0.13	-	16.85
G. kuroshium	*Female Bodies*	-	-	-	-	-
	Female Heads	15.33	-0.95	17.23	-0.15	-
	Larvae	-0.77	-17.82	0.24	1.24	-
	Males	17.12	-0.82	16.71	16.86	-
F. kuroshium + G. kuroshium	*Female Bodies*	-	-	-	-	-
	Female Heads	N/A	-13.27	-0.85	**2.65**	-
	Larvae	N/A	1.55	-0.34	**-1.86**	-
	Males	N/A	0.82	0.37	**1.1**	-

[a] Fungal treatment analysis done individually

[b] "-" indicates the reference for each multinomial model conducted for each treatment

[c] "N/A" represents cases where a particular species of fungi was not recovered

[d] Bold numbers indicate Wald-test significance at less than α=0.05

Table 3.4. Proportion table representing recovered fungal species from each beetle species and life stage by fungal treatment

			Recovered fungi from individuals				
Fungal treatment	**Beetle species**	**Sample type**	**Recovery proportion (%)[a]**				
			F. euwallaceae	*G. euwallaceae*	*P. pembeum*	*F. kuroshium*	*G. kuroshium*
Fusarium euwallaceae	*Euwallacea kuroshio*	*Female Bodies*	64.29	-	17.86	3.57	14.29
	E. kuroshio	*Female Heads*	97.54	-	-	-	2.46
	E. kuroshio	*Larvae*	36.84	-	-	-	63.16
	E. kuroshio	*Males*	74.32	-	6.76	1.35	17.57
Graphium euwallaceae	*E. kuroshio*	*Female Bodies*	-	86.36	-	-	13.64
	E. kuroshio	*Female Heads*	-	100.00	-	-	-
	E. kuroshio	*Larvae*	N/A	N/A	N/A	N/A	N/A
	E. kuroshio	*Males*	-	97.73	-	-	2.27
F. euwallaceae + G. euwallaceae	*E. kuroshio*	*Female Bodies*	16.67	54.17	25.00	4.17	-
	E. kuroshio	*Female Heads*	80.28	19.72	-	-	-
	E. kuroshio	*Larvae*	2.08	93.75	4.17	-	-
	E. kuroshio	*Males*	66.67	20.37	-	9.26	3.70
Paracremonium Pembeum	*E. kuroshio*	*Female Bodies*	-	-	100.00	-	-
	E. kuroshio	*Female Heads*	-	-	75.00	25.00	-
	E. kuroshio	*Larvae*	-	-	100.00	-	-
	E. kuroshio	*Males*	-	-	90.74	9.26	-
F. kuroshium	*E. kuroshio*	*Female Bodies*	-	-	57.14	42.86	-
	E. kuroshio	*Female Heads*	-	-	0.81	98.37	0.81
	E. kuroshio	*Larvae*	-	-	77.78	22.22	-
	E. kuroshio	*Males*	-	-	2.61	77.39	20.00
G. kuroshium	*E. kuroshio*	*Female Bodies*	-	-	-	-	100.00
	E. kuroshio	*Female Heads*	-	-	3.51	-	96.49
	E. kuroshio	*Larvae*	-	-	-	-	100.00
	E. kuroshio	*Males*	-	-	1.05	1.05	97.89
F. kuroshium + G. kuroshium	*E. kuroshio*	*Female Bodies*	-	-	2.17	15.22	82.61
	E. kuroshio	*Female Heads*	-	-	-	80.61	19.39
	E. kuroshio	*Larvae*	-	-	16.00	4.00	80.00
	E. kuroshio	*Males*	-	-	19.75	29.63	50.62
Fusarium euwallaceae	*E. whitfordiodendrus*	*Female Bodies*	93.33	2.22	4.44	-	-
	E.whitfordiodendrus	*Female Heads*	100.00	-	-	-	-
	E.whitfordiodendrus	*Larvae*	25.93	33.33	40.74	-	-
	E.whitfordiodendrus	*Males*	53.33	8.89	37.78	-	-
Graphium euwallaceae	*E.whitfordiodendrus*	*Female Bodies*	-	88.89	11.11	-	-
	E.whitfordiodendrus	*Female Heads*	11.90	84.52	3.57	-	-
	E.whitfordiodendrus	*Larvae*	-	100.00	-	-	-
	E.whitfordiodendrus	*Males*	1.63	91.87	6.50	-	-
F. euwallaceae + G. euwallaceae	*E.whitfordiodendrus*	*Female Bodies*	25.00	75.00	-	-	-
	E.whitfordiodendrus	*Female Heads*	98.25	1.75	-	-	-
	E.whitfordiodendrus	*Larvae*	2.41	95.18	2.41	-	-
	E.whitfordiodendrus	*Males*	33.33	64.65	2.02	-	-
Paracremonium Pembeum	*E.whitfordiodendrus*	*Female Bodies*	7.84	-	92.16	-	-
	E.whitfordiodendrus	*Female Heads*	10.67	-	89.33	-	-
	E.whitfordiodendrus	*Larvae*	-	10.00	90.00	-	-
	E.whitfordiodendrus	*Males*	1.04	6.25	92.71	-	-
F. kuroshium	*E.whitfordiodendrus*	*Female Bodies*	-	-	-	100.00	-
	E.whitfordiodendrus	*Female Heads*	-	-	-	100.00	-
	E.whitfordiodendrus	*Larvae*	-	-	67.86	32.14	-
	E.whitfordiodendrus	*Males*	-	1.45	31.88	66.67	-
G. kuroshium	*E.whitfordiodendrus*	*Female Bodies*	-	17.50	-	-	82.50
	E.whitfordiodendrus	*Female Heads*	4.76	7.14	-	-	88.10
	E.whitfordiodendrus	*Larvae*	-	-	-	-	100.00
	E.whitfordiodendrus	*Males*	22.83	6.52	1.09	-	69.57
F. kuroshium + G. kuroshium	*E.whitfordiodendrus*	*Female Bodies*	-	1.19	14.29	27.38	57.14
	E.whitfordiodendrus	*Female Heads*	-	-	1.99	81.46	16.56
	E.whitfordiodendrus	*Larvae*	-	7.06	4.71	4.71	83.53
	E.whitfordiodendrus	*Males*	-	1.71	3.42	54.70	40.17

[a] "N/A" represents cases where individuals were not sampled

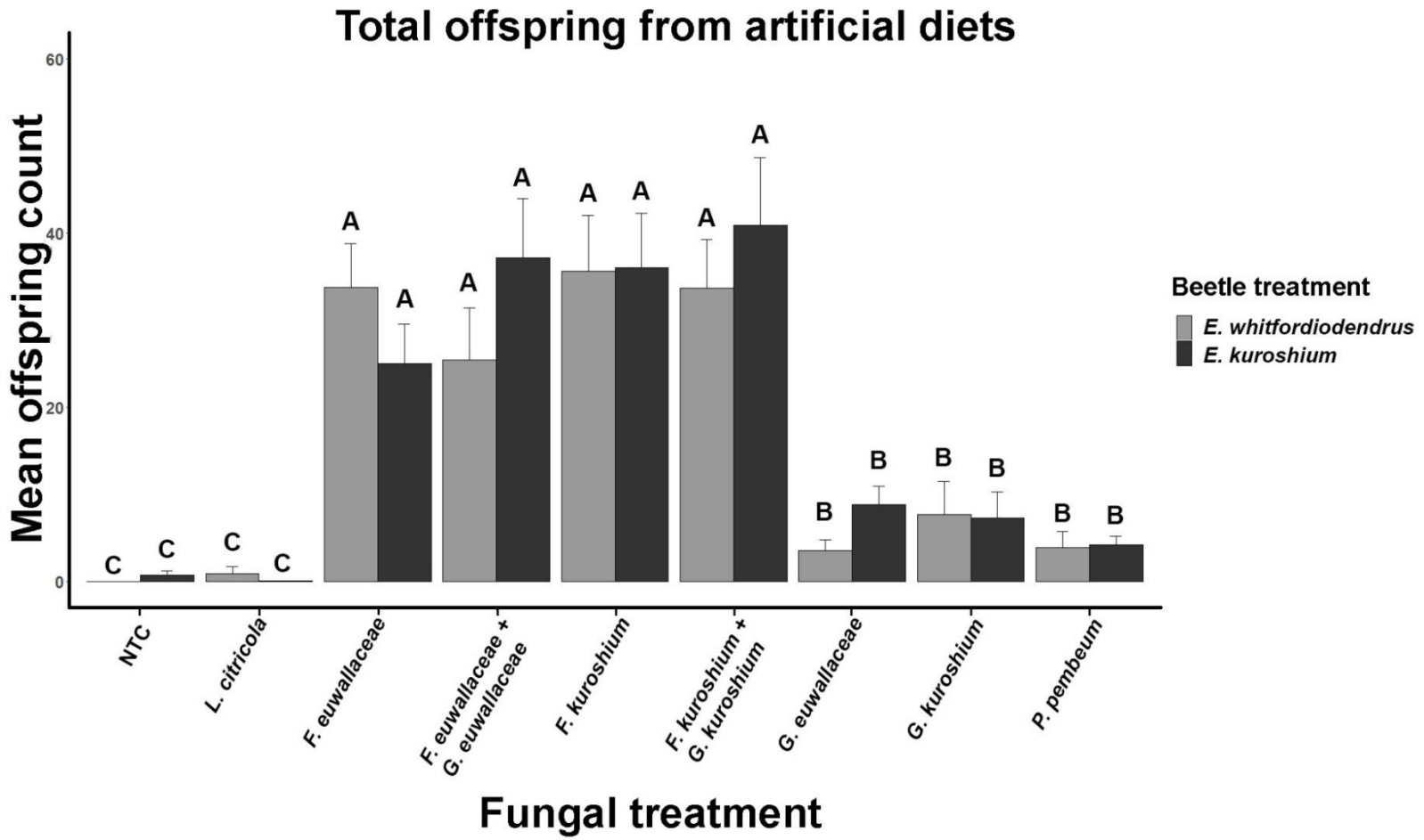

Figure 3.1. Total offspring counts from all individuals within each fungal treatment group. Letters represent significant contrasts at α=0.05 using post-hoc EMM from negative binomial regression model

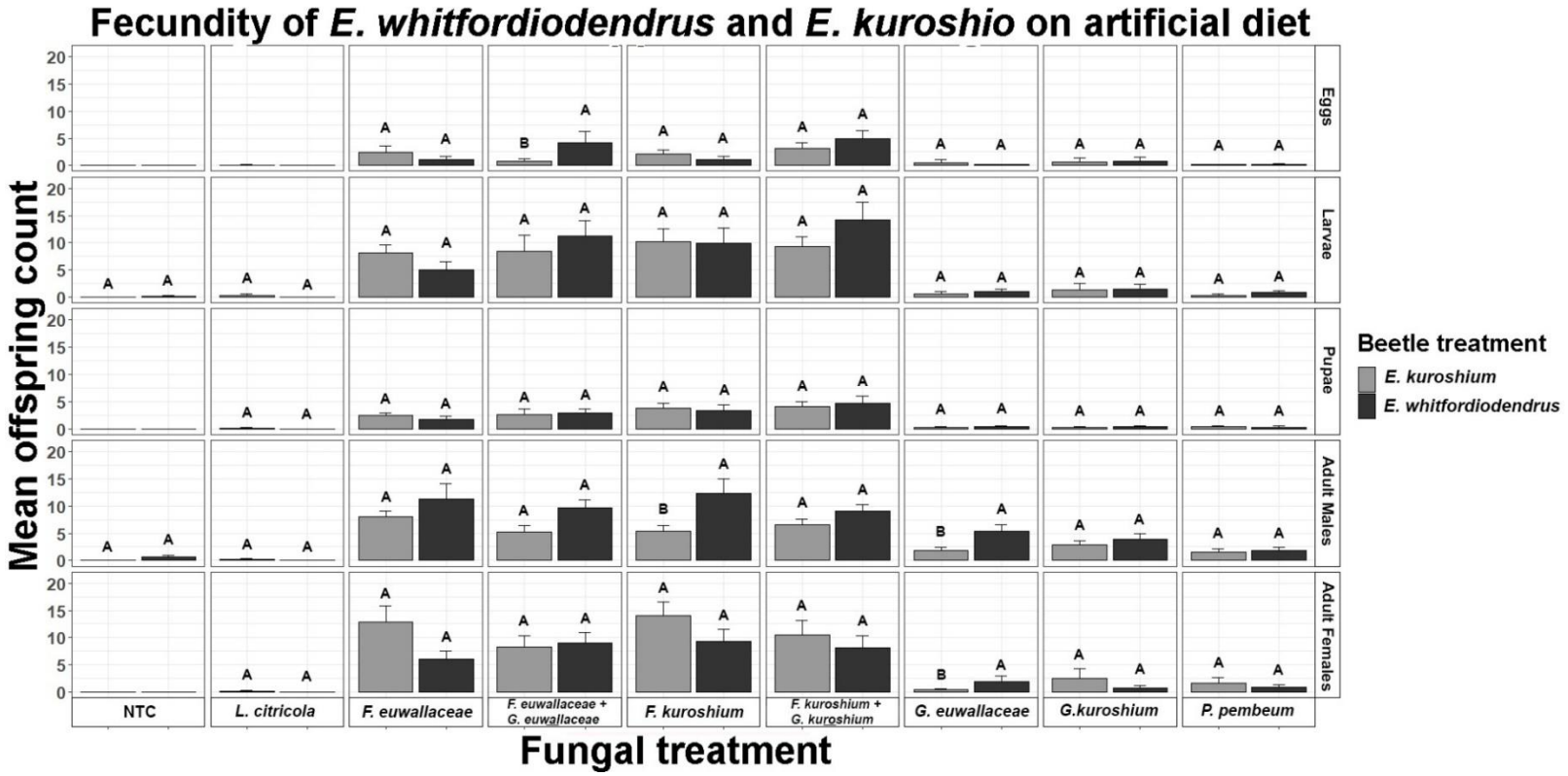

Figure 3.2. Offspring counts split by sample type within each fungal treatment group. Letters represent significant contrasts within each facet at α=0.05 using post-hoc EMM from negative binomial regression model

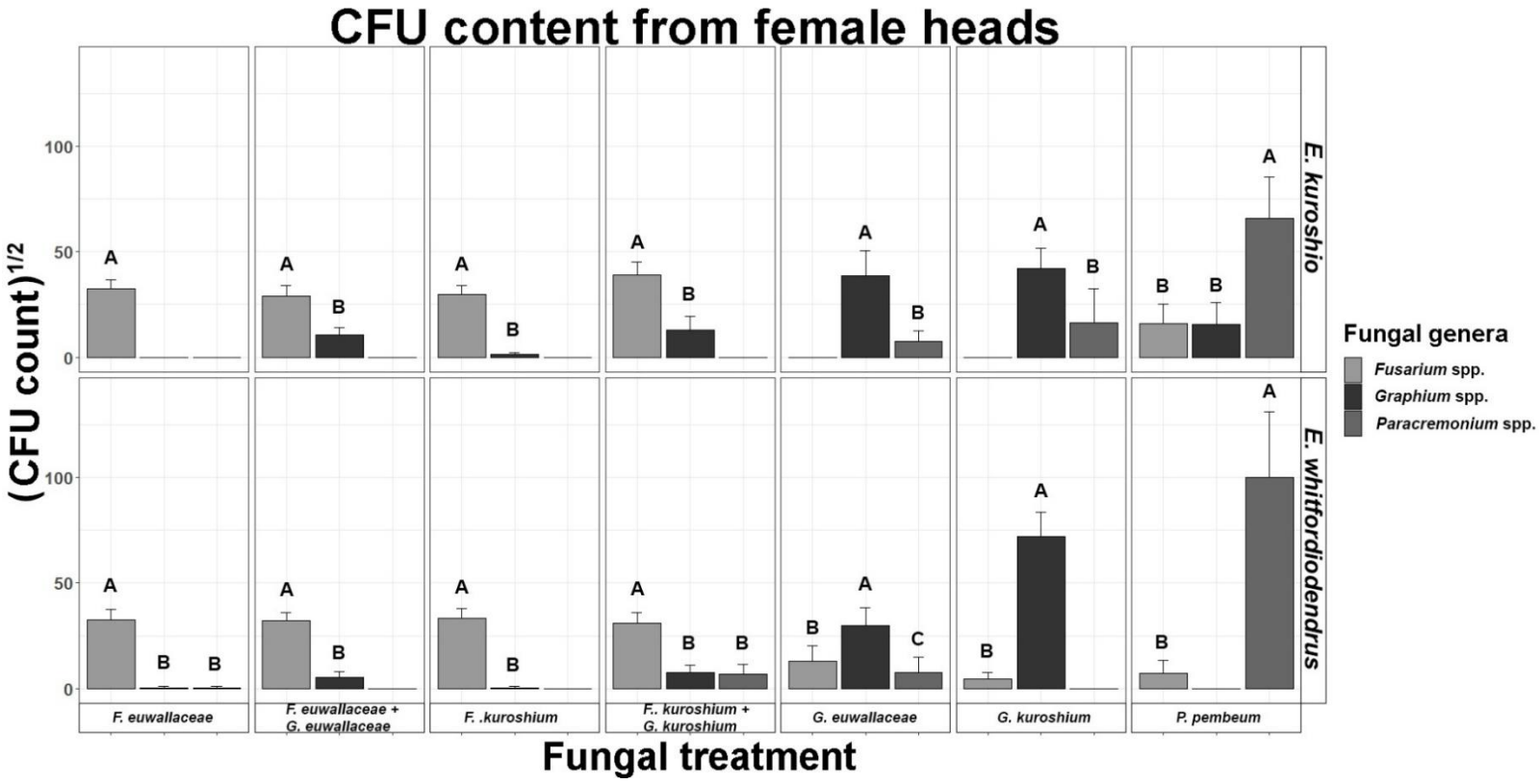

Figure 3.3.1. Total CFU counts recovered from female heads from each respective fungal genus separated by beetle species. Letters represent significant contrasts within each facet at α=0.05 using post-hoc EMM from Poisson regression model

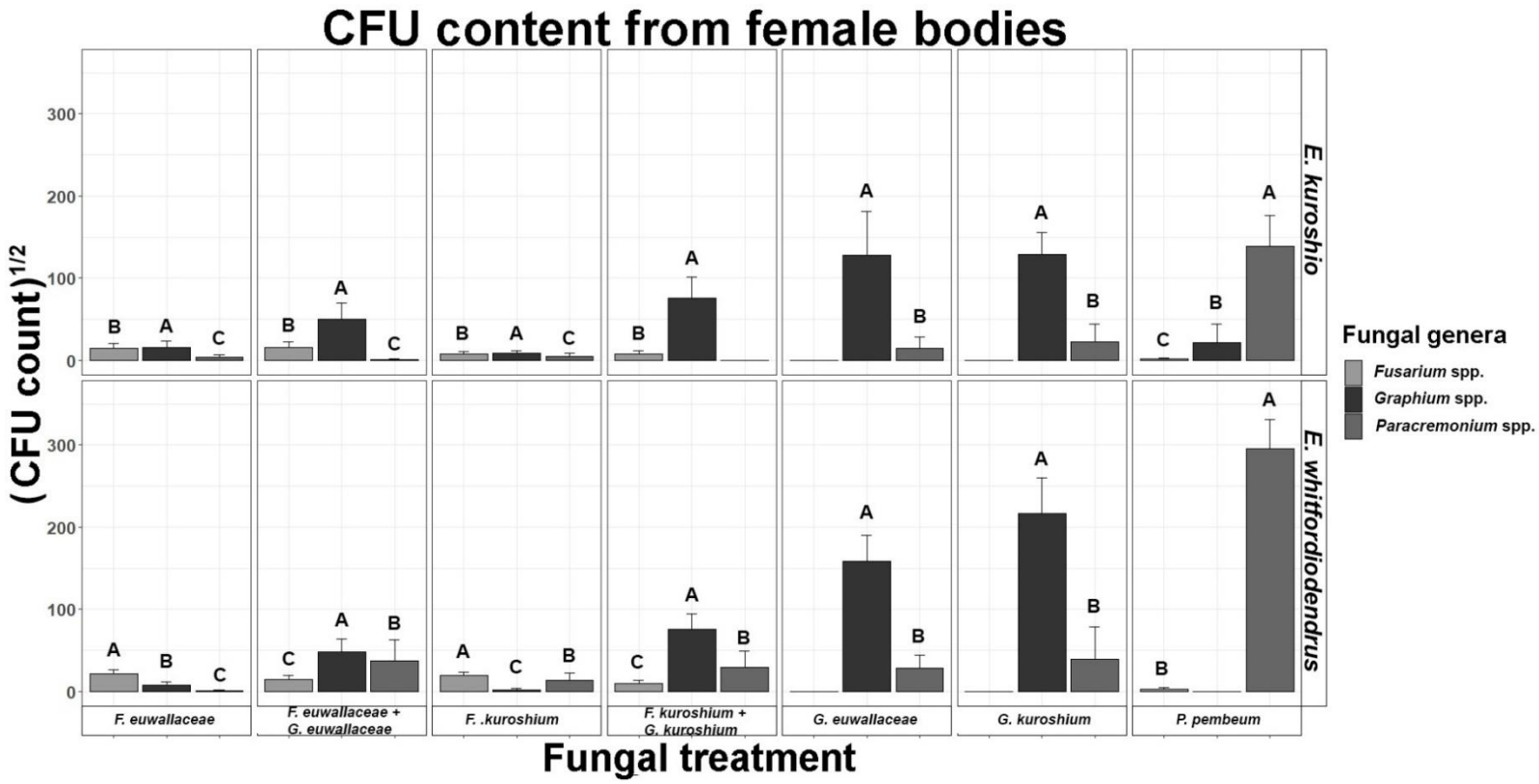

Figure 3.3.2. Total CFU counts recovered from female bodies from respective fungal genera separated by beetle species. Letters represent significant contrasts within each facet at α=0.05 using post-hoc EMM from Poisson regression model

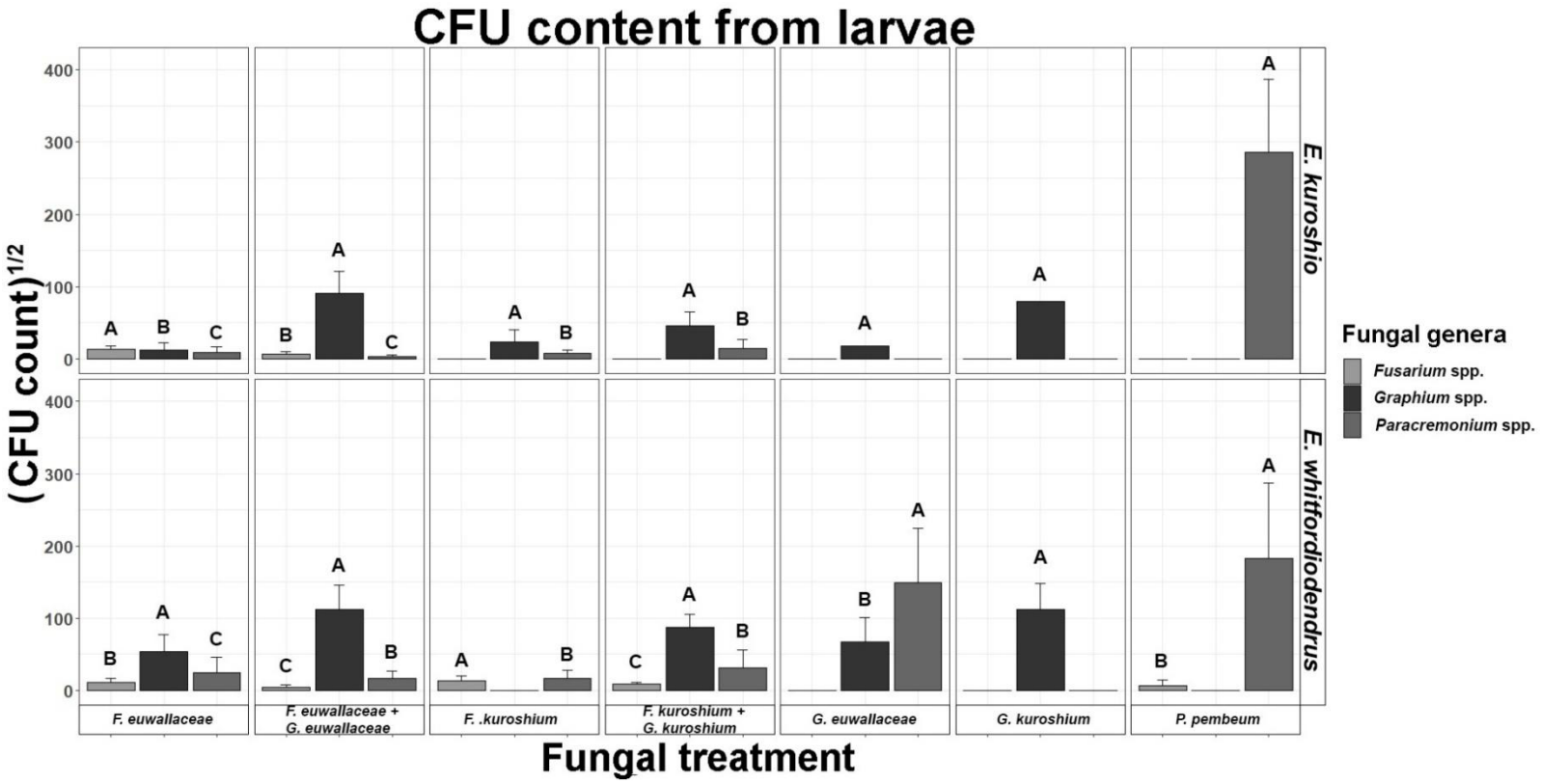

Figure 3.3.3. Total CFU counts recovered from larvae from each respective fungal genus separated by beetle species. Letters represent significant contrasts within each facet at α=0.05 using post-hoc EMM from Poisson regression model

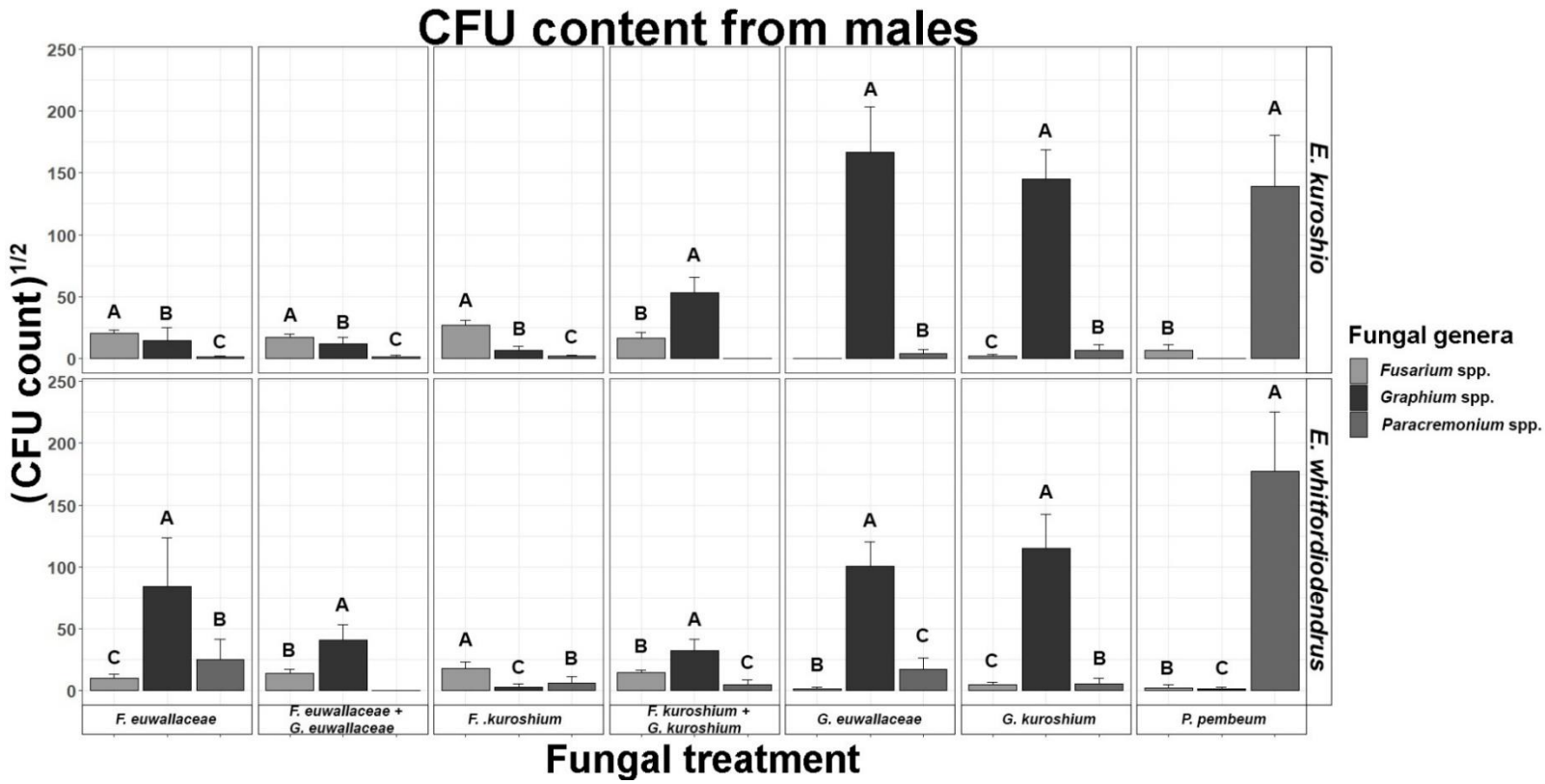

Figure 3.3.4. Total CFU counts recovered from males from respective fungal genera separated by beetle species. Letters represent significant contrasts within each facet at α=0.05 using post-hoc EMM from Poisson regression model

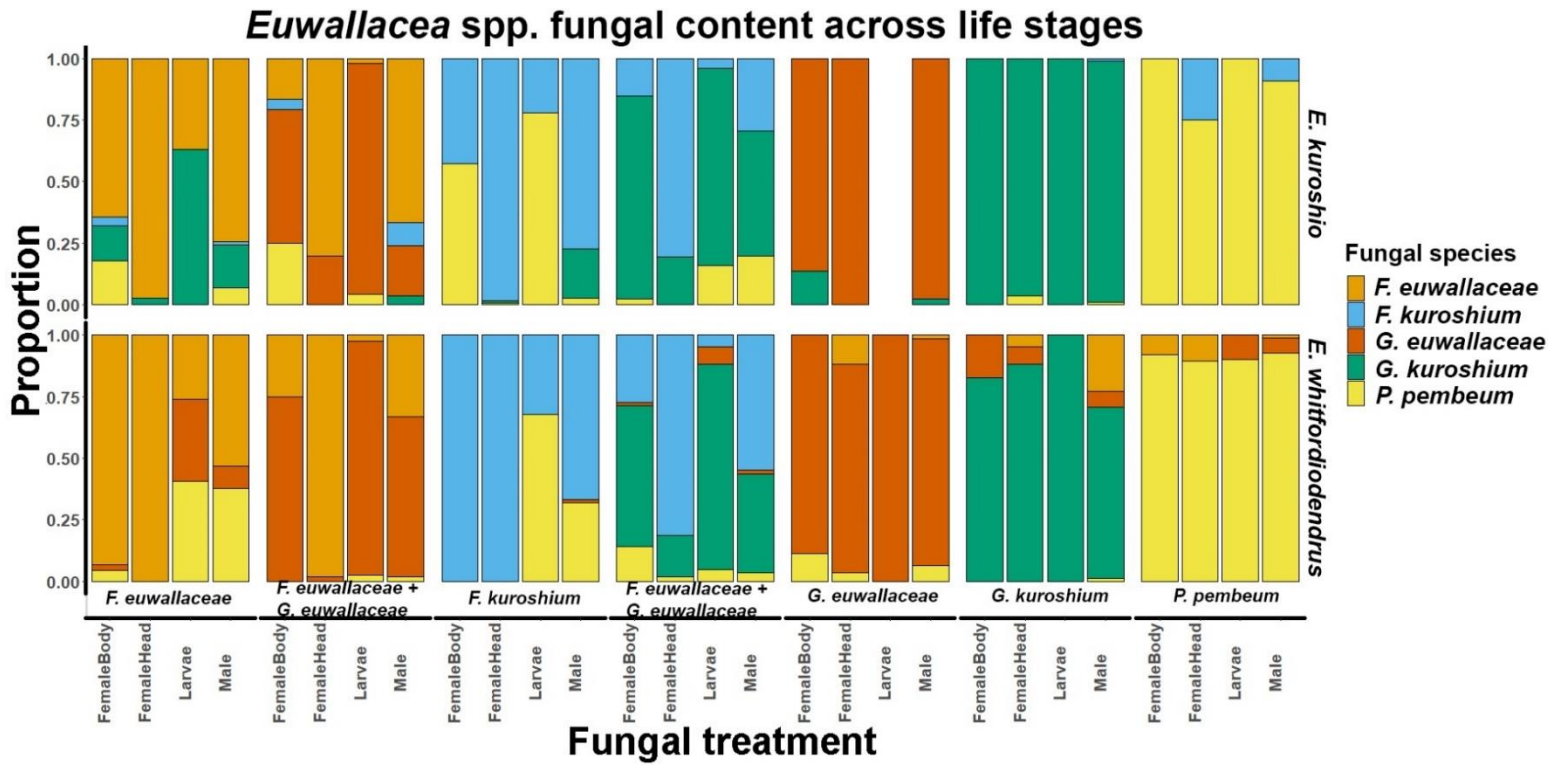

Figure 3.4. Proportions of fungal species within each fungal treatment group faceted by sample types within beetle species given different fungal diet treatments

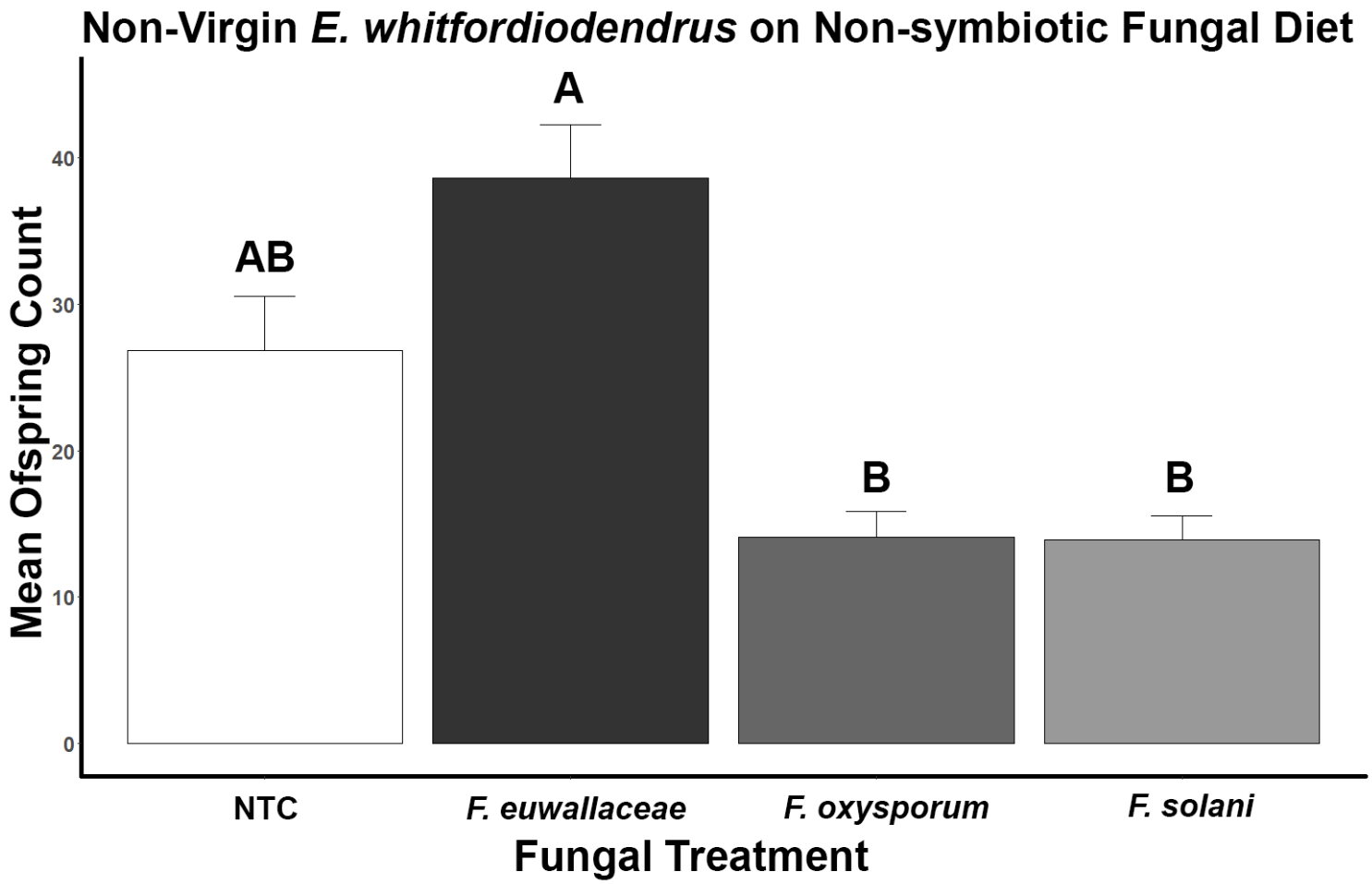

Figure 3.5.1. Total offspring counts from all individuals within each fungal treatment group with non-virgin females. Letters represent significant contrasts at α=0.05 using post-hoc EMM from negative binomial regression model

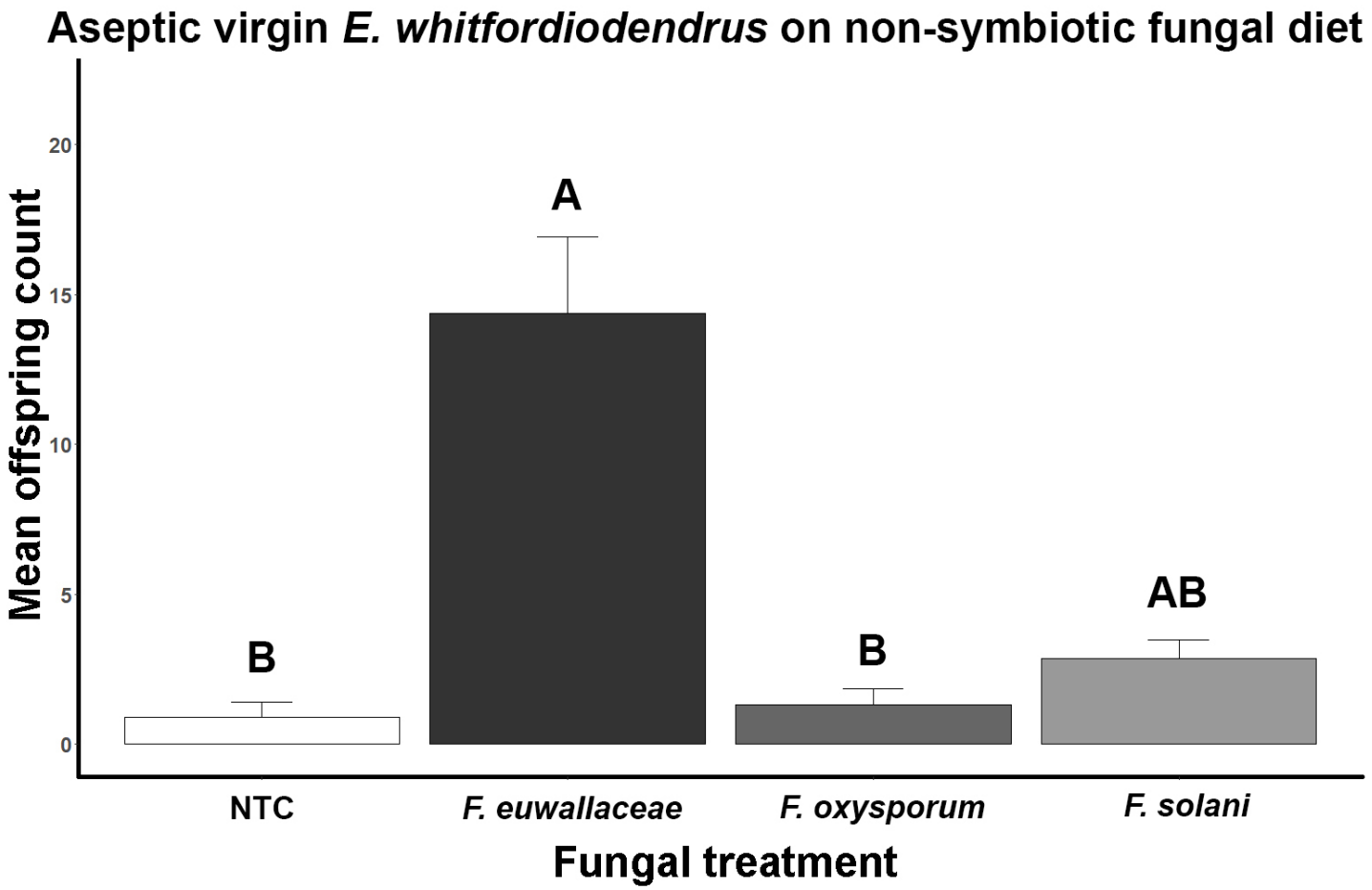

Figure 3.5.2. Total offspring counts from all individuals within each fungal treatment group with aseptic virgin females. Letters represent significant contrasts at $\alpha=0.05$ using post-hoc EMM from negative binomial regression model

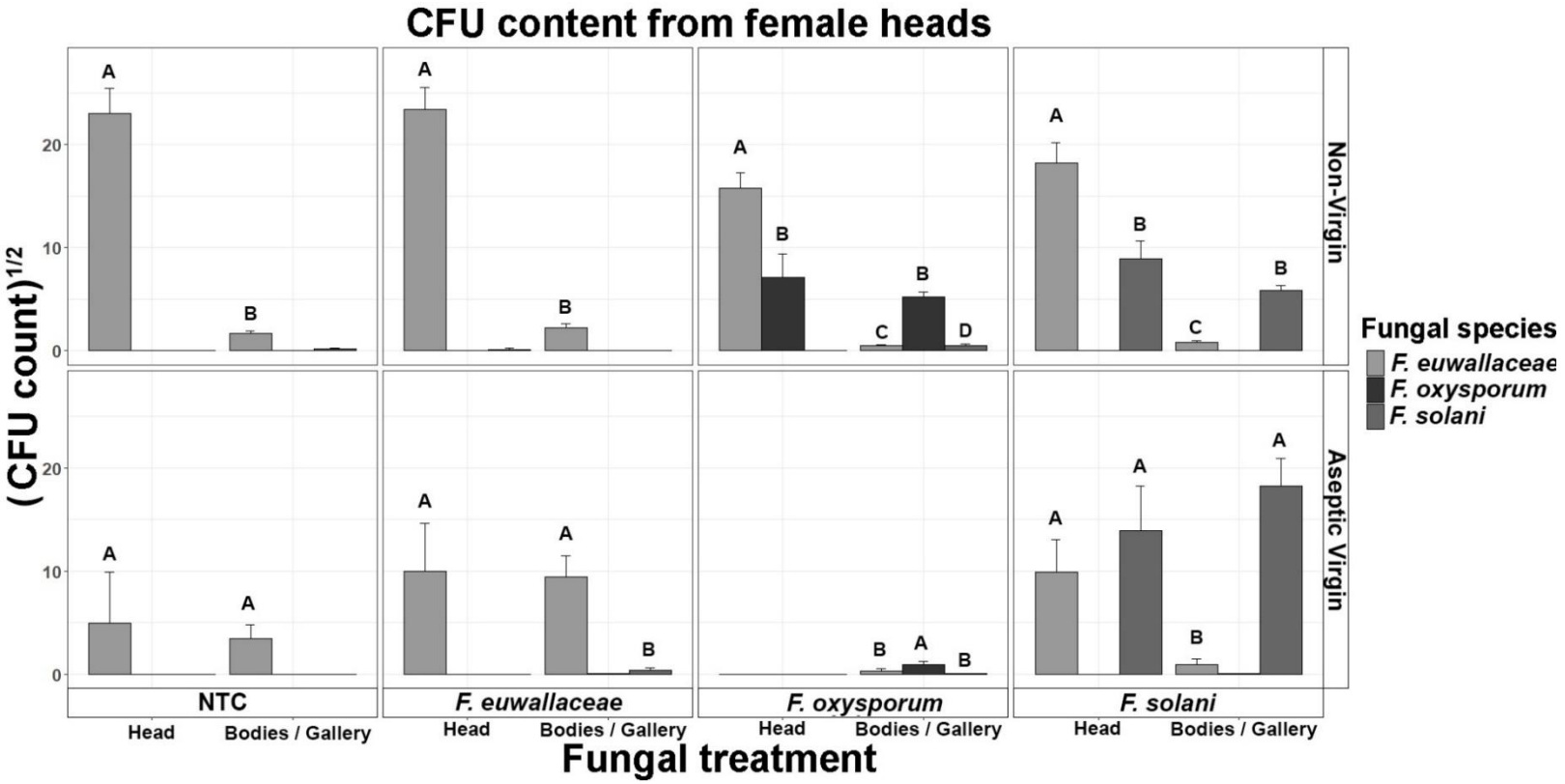

Figure 3.6. Total CFU counts recovered from the non-symbiont trial from each respective fungal species separated by virginity status. Letters represent significant contrasts within each facet at α=0.05 using post-hoc EMM from Poisson regression model

CHAPTER IV. Integrated Pest Management of Polyphagous Shot Hole Borer in Avocado (*Persea americana*) in California

ABSTRACT

Invasive ambrosia beetles *(Euwallacea* spp.) commonly called shot hole borers (SHB) have been posing a threat to avocado groves as well as urban landscape and wildlands in California. Previous studies show that SHB tend to colonize primary, secondary and tertiary branches including branch collars of avocado. Therefore, effective management strategies for commercial avocado groves are limited to removal of infested branches to reduce beetle populations. However, some beetle galleries still remain around the branch collar which support future beetle reproduction and spread This investigation into pesticides for use in avocado against California invasive pest *E. whitfordiodendrus* commonly known as the polyphagous shot hole borer (PSHB) and FD indicate pesticide candidates have significant effectiveness against PSHB and their fungal symbionts *in vitro* and in field trials. The most effective treatment across all experimental trials was found to be the contact insecticide bifenthrin/ zeta-cypermethrin. Integrative pest management combining cultural management through phytosanitation along with chemical management can aid in reducing local PSHB populations within avocado groves, help reduce disease pressure and help prevent further spread within avocado groves as well as to other areas.

INTRODUCTION

Invasive ambrosia beetles (*Euwallacea* spp.) were first discovered attacking apparently healthy looking tree species in Los Angeles County in 2012 (Eskalen et al. 2013) and San Diego County in 2013 (Stouthamer et al. 2017) and have since caused

extensive damage to tree species in wildlands, urban landscapes, and commercial avocado groves in southern California. Most ambrosia beetle species are known to infest and breed in dying or decaying trees by feeding on their ambrosia fungi (Beaver 1989), but in invaded areas, such as California, two *Euwallacea* spp. have been found to successfully colonize and cause mortality (in severe cases) in apparently healthy trees (Hulcr and Stelinski 2017). Repeated attacks by the beetles and inoculation of ambrosia fungi within the host leads to a disease known as Fusarium Dieback (FD, Eskalen et al. 2012). This disease is the result of colonization from symbiotic fungi cultivated by the beetles as they bore within the woody tissue of the host. The most obvious symptoms on avocado are discoloration in areas of the outer bark surrounding the point of attack (0.85 mm diameter) which is usually covered by a large amount of the white powdery exudate which is a form of sugar termed 'perseitol'(aka "sugar volcano") (Mendel et al. 2017). In severe cases of infestation, dieback of branches can be visible in heavily infested areas which generally occur on primary, secondary, or tertiary branches with occasional attacks on the trunk (Lynch et al. 2018). Additional symptoms include wilting of branches, discoloration of leaves in some cases, breakdown of branches from fungal colonization and extensive tunneling by the beetle within branches (Mendel et al. 2017).

In California there are two similar but distinct species of invasive shot hole borers (SHB) present: *E. whitfordiodendrus* Schedl and *E. kuroshio* Gomez and Hulcr. *E. whitfordiodendrus* commonly known as the Polyphagous shot hole borer (PSHB) is associated with distinct fungal symbionts including *F. euwallaceae* S. Freeman, Z. Mendel, T. Aoki & O'Donnell, in addition to *Graphium euwallaceae* M. Twizeyimana,

S.C. Lynch & A. Eskalen, and *Paracremonium pembeum* S.C. Lynch & Eskalen (Eskalen et al. 2013; Lynch et al. 2016; Mendel et al. 2012). *E. kuroshio*, commonly known as the Kuroshio shot hole borer (KSHB), is associated with *Fusarium kuroshium* F. Na, J. D. Carrillo & A. Eskalen and *Graphium kuroshium* F. Na, J. D. Carrillo & A. Eskalen (Na et al. 2017). All of the fungi associated with PSHB and KSHB have been previously confirmed as plant pathogens (Lynch et al. 2016; Mendel et al. 2012; Na et al. 2017), with a current host range of sixty-four hardwood species they can reproduce in, which are abundant in areas adjacent to many avocado groves.

The PSHB invasion is not unique to California and has been reported invading other areas including Israel (Freeman et al. 2012), and South Africa (Paap et al. 2018) where they have been detected causing damage in avocado groves in California and Isreal, as well tree species in urban forest in all invaded areas. In California, the economic burden of the pests, specifically on avocado, is unknown but in the urban landscape, PSHB has infested one-third of California sycamore (*Platanus racemosa*) in Orange County public parks and caused the removal of 1262 trees, resulting in approximately $4 million USD in tree removal costs (OC Parks 2016). Within avocado groves, a recent survey found 80% of 18 surveyed groves were positive for beetle infestation in San Diego and Orange Counties, while in Ventura county 14 percent of 35 surveyed groves were found positive with the latter number expected to increase as the beetles spread northward (Lynch et al. 2018). California is the largest producer of avocados in the U.S. with over 50,000 bearing acres at an estimated value of $345 million USD (California Avocado Commission 2018). Since these pests are already widely present in major

growing areas in southern California, they pose a viable threat for avocado growers in California.

Currently, there are limited management strategies to control these pests in commercial avocado groves, urban landscapes and wildlands in California. Common proposed methods to manage beetle populations include removal of infested wood, subsequent solarization or chipping, and in severe cases tree removal (Eatough-Jones and Paine 2015). Removal of infested branches has recently been suggested as a method of management to reduce the beetle population in groves (Mendel et al 2017, Lynch et al 2018). However, SHB have been observed colonizing areas that cannot be removed by regular pruning, such as the branch collar, which makes SHB difficult to reduce beetle populations in an avocado grove. In addition, SHB can be active year-round in an infected avocado grove (Eatough-Jones and Paine 2015) and can complete their life cycle within 22 days (Cooperband et al. 2016). Exploring the potential commercially available pesticides to control the beetle in avocado groves could provide and effective control option in addition to cultural management practices that have been recommended so far (Mendel et al 2017, Lynch et al 2018).

Since the beetles are dependent on their ambrosia fungi for survival (Beaver 1989), we decided to investigate more for treatments targeting both vector and pathogen in this study. While no commercial insecticides registered on avocado were available to control PSHB, in 2016 a chemical insecticide premix of bifenthrin (pyrethroid) and zeta-cypermethrin (pyrethroid) was registered temporarily under emergency Section 18 specific exemption but has since expired (California Avocado Commission 2018).

Systemic pesticide treatments including emamectin benzoate and bifenthrin (pyrethroid insecticides) and a combination of emamectin benzoate and propiconazole (triazole fungicide) were all found to significantly reduce PSHB infestation in California sycamore (Mayorquin et al. 2018), however, these pesticides are not currently registered on avocado. Among fungicides that are currently registered for use other fungal diseases on avocado, azoxystrobin (strobilurin) has been found to significantly reduce the growth of all fungal symbionts associated with PSHB *in vitro* (Mayorquin et al. 2018). Some biological control agents have been shown to be effective against PSHB and its fungal symbionts and are commercially available for use in avocado. *B. subtilis* has been reported to cause mycelial inhibition when plated against PSHB fungal symbionts *in vitro* (Guevara-Avendaño et al. 2018) while *Beauveria bassiana* has been shown to be effective in colonizing Scolytine ambrosia beetles *Xylosandrus germanus*, a close relative to PSHB (Castrillo et al. 2011)). Investigation into the efficacy of both chemical and biological pesticides to combat these invasive pests could provide both conventional and organic avocado growers additional management strategies regardless of the operation type.

This study was initiated to investigate effective management options for avocado growers currently combating these pests in California. Therefore, alternative pesticide treatment options in addition to cultural management (phytosanitation) should be considered to reduce PSHB populations in avocado groves . The objective of this study was to investigate the efficacy of various chemical fungicides, an insecticide, and biological control agents by (i) performing *in vitro* assays evaluating pesticide efficacy

against the ambrosia fungi associated with PSHB and KSHB; (ii) investigating effects on PSHB fecundity by amending sawdust rearing media with pesticide treatments; (iii) performing *in vitro* bioassays with treated avocado wood bolts; and (iv) conducting field trials by controlling introductions of PSHB to spray treated avocado branches using 3D printed traps.

METHODS

Preparation of fungicide and *Bacillus subtilis* amended culture media. Potato Dextrose Agar (PDA; BD Difco, Sparks, MD, USA) amended with commercially available fungicides including azoxystrobin (Abound®, 2700ppm AI), thiabendazole (Arbortect®, 50ppm AI), and 30% (v/v) for *B. subtilis* (Serenade® ASO) (Table 1). at max labeled rate were used for culture media. All fungicide amendments were added after the autoclave when the media had cooled to 60°C. Ten milliliters of molten media was aliquoted onto petri dishes, allowed to solidify, and radial disks from pure cultures of SHB symbionts *F. euwallaceae* (NRRL62626, UCR4920), *G. euwallaceae* (CBS 140035, CBS140034), *P. pembeum* (CBS 140042, UCR2983), *F. kuroshium* (CBS142642, UCR3644), and *G. kuroshium* (CBS142643, UCR4593) were made using a 3mm cork-borer and placed on the designated amended media types. Media were incubated at 25°C for 10 days and radial measurements were taken at 3 and 10 days.

***In vitro* amendment to sawdust rearing media.** Sawdust rearing media (75 g avocado sawdust, 20 g agar, 10 g sucrose, 5 g casein, 5 g starch, 5 g yeast, 1 g Wesson's salt mixture, 2.5 ml wheat germ oil, and 5 ml 95% ethanol in 500 ml of distilled water) modified from Norris and Chu (1985) was used to introduce SHB directly to the various

pesticide treatments mixed in the media. All sawdust rearing media treatments were amended with streptomycin (0.35g/L) prior to autoclaving, except for *B. subtilis* treatment without streptomyocin. Pesticides were added after media had cooled to 60°C and mixed with a sterilized paint mixer attached to a standard power drill. Twenty milliliters of media was poured into sterile 50mL centrifuge tubes and once cooled, two starter holes were created with a 1.5 mm sterile drill bit before a single, inseminated PSHB female was introduced into the rearing media. Treatments included 2700 ppm azoxystrobin (Abound®), 50ppm thiabendazole (Arbortect 20S®), 262ppm bifenthrin/ zeta-cypermethrin (Hero®), 30 percent (v/v) *Bacilus subtilis* (Serenade® ASO) (autoclaved), a non-antibiotic amended sawdust media inoculated with 10 µl diluted *B. subtilis* (Serenade® ASO) (30% v/v), 10 µl of $9x10^{10}$ spores/ml *Beauveria bassiana* (Mycotrol WPO®), and a non-treated control (NTC). Ten replicates were prepared for each treatment and previously reared inseminated female beetles were surface disinfested with 30% ethanol and two sterile water washes then allowed to dry on sterile filter paper. The females were introduced to the treated media and total offspring were counted after 40 days. Any females present from surviving treatments were sampled and the offspring samples were surface sterilized by immersion in 70% ethanol and vortexed for 10 s, rinsed with sterile de-ionized water, and allowed to dry on sterile filter paper. All samples were processed individually and adult female beetle heads were separated from the thoracic and abdominal segments under a dissection microscope then processed separately to determine the fungal contents of the female head and female body. All samples were macerated in 1.5 ml microcentrifuge tubes with sterile plastic pestles.

Additional sampling of the gallery wall with a sterile toothpick was performed. To sample gallery walls, the media was opened to expose the inner gallery wall and the tip of a toothpick was rotated 360° in the gallery then suspended in 1 ml sterile 0.01% triton. The macerated female samples were suspended in 1 ml of sterile 0.01% triton (adult female bodies suspended in 200 ul) and further diluted at different rates depending on the sample type: 1:1 dilution for female heads and no dilution for female bodies or gallery samples. Twenty-five microliters of suspension from each sample was pipetted onto two petri dishes containing PDA amended with tetracycline hydrochloride (0.01%) and spread using sterile glass L-shaped rods. The PDA plates were incubated at 25°C for one week and colony forming units (CFU) were counted. *F. euwallaceae*, *G. euwallaceae*, and *P. pembeum* were distinguished based on previous descriptions of morphology on PDA (Freeman et al 2013; Lynch et al; 2016).

PSHB introduction to pesticide treated avocado bolts. Avocado wood (~ 5 cm diameter) from the "Hass" cultivar at University of California Riverside (UCR) Agricultural Operations was collected, then cross sectionally cut into 8 cm pieces to represent a "pruned" surface (Fig 1B) and additional wood was also cut both cross sectionally at 11 cm length *then* longitudinally to represent a "bark" introduction (Fig. 1A) and give a flat bottom surface to rest in individual wide mouth mason jars. The cut ends not receiving pesticide treatment were sealed with paraffin wax. Treatments were identical to those used in sawdust media except a water treated control was used in place of a non-treated control and all treatments were applied with a hand-held spray bottle until runoff and allowed to air dry. Five of each prepared "pruned" and "bark" pieces

were treated. Brass compression rings were used to create small chambers to keep the beetles on the wood pieces. Four young inseminated females were introduced to each ring chamber on each treated wood bolt and glass coverslips with a small amount of petroleum jelly in the shape of the ring were placed over top to prevent beetle escape from the ring chamber. The wood bolts were placed into designated wide mouth mason jars with a screen lid lined with filter paper then placed in plastic containers elevated on plastic lighting diffusers lined with paper towels soaked with deionized water to maintain humidity for 40 days at room temperature. Randomized complete block design (RCBD) was used and with five humid containers representing a block containing one replication from six treatments and each branch type within. The beetles were obtained from sawdust rearing media and were randomly assigned to one of the six treatments for both branch types. This experiment was repeated twice After 40 days, wood was destructively sampled using a bandsaw to evaluate the brood gallery success and presence of offspring in the wood bolts and entry ratios were calculated from the amount of entry holes present divided by the four females introduced. Gallery success (Fig 1C) was determined if tunnels were greater than 10 mm length.

Effective rates of insecticide on PSHB using avocado bolts. Avocado bolts were prepared as mentioned above for the “bark” setup (Figure 1A) and different rates of bifenthrin/ zeta-cypermethrin (Hero®) were applied until runoff with a hand-held sprayer and allowed to air dry completely. Each bolt was assigned 4 compression rings in which 4 beetles were introduced into each chamber as mentioned above then placed into the mason jar with a screened lid and incubated in one humid chamber under the same

conditions as described above. The treatments included 10ppm, 25ppm, 50ppm, 100ppm, and 262ppm bifenthrin/ zeta-cypermethrin (Hero®) with each treatment having two replicates for a total of 32 observations per rate. Over five days, the beetles were monitored and gallery success was evaluated at the end of the fifth day.

Field PSHB introduction. Locations in Riverside (UCR Agricultural Operations) and Pomona (Cal Poly Pomona, CPP) were being used to introduce live lab reared inseminated PSHB females into treated avocado branches from May 2018 to August 2018. These months were chosen based on previous studies which show increased beetle activity in warmer months (Lynch et al. 2018; Mayorquin et al. 2018). Temperature and relative humidity were monitored using a HOBO weather station with a solar shield (HOBO, Onset Compute Corp., MA, USA). Avocado trees at UCR were "cv. Hass" cultivar on "Dusa" rootstock and less than ten years old while avocados at CPP were "cv. Fuerte" cultivar on an unknown rootstock with age greater than ten years old. Within each plot, the experimental design was done by RCBD with each treatment being randomized on branches within each tree (block) on five trees per location over two trials. Selected branches were spray treated until runoff with either 2700 ppm azoxystrobin (Abound®), 50ppm thiabendazole (Arbortect 20S®), 262ppm bifenthrin/ zeta-cypermethrin (Hero®), 30 percent (v/v) *B. subtilis* (Serenade® ASO), a solution of $9x10^{10}$ spores/ml *B. bassiana* (Mycotrol WPO®), and an untreated control. Twenty-four hours post-application, four beetles were introduced using 3D printed traps (Figure 1D) (2 beetles per branch) and fastened to the tree with steel screws to secure the traps. After

40 days the infested wood was removed to evaluate the entry ratio of the 4 beetles, as well as the gallery success if tunnels were greater than 10 mm in length.

Statistical analyses. All statistical analyses were performed using R (Version 3.4.3). *In vitro* radial growth measurements were converted to percent inhibition based on the control and all treatments were compared using linear regression. Homoscedasticity for all independent, *in vitro* runs for each fungal species tested was evaluated using Levene's test; no heterogeneity was detected and thus data from isolates of the same species were combined. Analysis of Variance (ANOVA) was run to compare independent variables using the *"anova* function while Tukey contrasts were used to compare treatments using the "multcomp" package (Hothorn et al. 2008). Due to the excess zero counts and over-dispersion of both offspring count data sets, as the variance exceeds the mean, negative binomial regression analysis using a generalized linear model was performed using the "MASS" package (Venables and Ripley, 2002). ANOVA was used compare independent variables in the regression model using the *anova* function. Pairwise comparisons were done across all treatments for total offspring counts using estimated marginal means (EMM) with the "emmeans" package (Lenth et al. 2017). For CFU spore count data from females and gallery samples was done with Poisson regression analysis using a generalized linear model with the "MASS" package (Venables and Ripley 2002). Post-hoc analysis for CFU data was done by constructing a contrast matrix in order to compare all responses to all treatments by sample type, then by using the "multcomp" package (Hothorn et al. 2008) with link function *glht* for comparison of fungal recovery CFU counts across all treatments based on the matrix. For both the bolt and field trial entry

ratio and gallery success were separately analyzed using logistic regression analysis with a binomial distribution using the "MASS" package (Venables and Ripley, 2002). Pairwise comparisons were done across all treatments for the respective response variables using EMM with the "emmeans" package (Lenth et al. 2017). All bar plots were generated using "ggplot2" (Wickham 2016)

RESULTS

***In vitro* fungicide amendment to culture media.** No significant differences were observed between isolates ($P > 0.05$) of the same species therefore *post hoc* analysis was carried by grouping the isolates by their respective species. There were significant differences ($P < 0.05$) among fungicides tested *in vitro* against the fungal symbionts of SHBs (Fig. 2). Azoxystrobin applied at the maximum field label rate did not completely inhibit the growth of all symbionts but significantly reduced mycelial growth compared to the control across all species tested ($P < 0.05$), where all species except the *Fusarium* spp. growth was inhibited by over 50% (Fig. 2). Media amended with the maximum label rate of autoclaved *B. subtilis* and 50ppm thiabendazole both completely inhibited growth of all symbionts and showed significantly greater inhibition that that of the maximum label rate azoxystrobin (Fig. 2). It should be noted that max rate bifenthrin/ zeta-cypermethrin (Hero®, 262 ppm AI) was tested but was not included in the analysis due to the lack of inhibition.

PSHB fecundity in pesticide amended sawdust media. Mean total offspring counts across all pesticide treatments were significantly different ($P < 0.05$) with most offspring produced on the NTC. Media amended with azoxystrobin, thiabendazole, and *B. subtilis*

without streptomycin produced significantly less offspring than the untreated control, but significantly more than zeta-cypermethrin, autoclaved *B. subtilis* with streptomycin, and *B. bassiana*, which all produced no offspring (Fig. 3). Mycosed beetles were observed in *B. bassiana* treatments. When offspring were separated into female, male, and larvae groups, treatments that produced offspring had significantly less ($P < 0.05$) larvae compared to females compared to the NTC (Fig. 4). In azoxystrobin and *B. subtilis* without streptomycin treatments that produced offspring, females were dominant. (Fig. 4). CFU counts within individual female mycangia, gut, and gallery samples showed significant effects ($P < 0.05$) on recovered fungi by sample type within each pesticide treatment (Fig. 5). Across all pesticide treatments that produced offspring, female heads contained significantly more *F. euwallaceae* compared to the other symbionts in all treatments except thiabendazole in which females contained a large amount of *P. pembeum* not significantly different ($P > 0.05$) from *F. euwallaceae.* This was also observed in the *B. subtilis* treatment without streptomycin, where *P. pembeum* CFU counts were higher in the female heads but significantly lower ($P < 0.05$) than CFU counts of *F. euwallaceae.* Recovery of *P. pembeum* within the thiabendazole and *B. subtilis* without streptomycin treatments from female gut and gallery samples was significantly higher ($P < 0.05$) when compared to other fungal symbionts. CFU recovery from azoxystrobin-amended media shared a similar profile with the untreated control.

PSHB success on pesticide treated avocado bolts. No significant differences were observed between “bark” and “pruned” type bolts, however the analysis for both bolt types were carried out individually. Entry ratios were significantly reduced in the

insecticide treatment bifenthrin/ zeta-cypermethrin as no beetles successfully entered the bolts. Within the "bark" group there were no significant differences in entry ratio between treatments aside from bifenthrin/ zeta-cypermethrin (Fig. 6A). Within the "pruned" group there were significant differences with bifenthrin/ zeta-cypermethrin, *Beauveria bassiana*, azoxystrobin, and thiabendazole with lower entry ratios when compared with the water treated control group. Gallery success within the "bark" group treated with bifenthrin/ zeta-cypermethrin and thiabendazole had significantly lower success than the control (Fig. 6B). Within the "pruned" group, bifenthrin/ zeta-cypermethrin, thiabendazole, *Beauveria bassiana*, and azoxystrobin gallery success was significantly lower than the control (Fig. 6B). When testing different insecticide concentrations, significant differences in gallery success were observed across application rates of bifenthrin/ zeta-cypermethrin indicating increased rates had effects on PSHB ability to colonize the wood (Fig. 7). The maximum label rate of bifenthrin/ zeta-cypermethrin resulted in no successful galleries as beetles were observed to be dead twenty-four hours post treatment.

Field PSHB introduction. Temperature and humidity levels between the two field sites was not significantly different over the duration of the experiment (Data not shown). The UCR and CPP field sites were analyzed separately due to the difference in cultivar and age of the trees although there was no significant difference ($P < 0.05$) between the two data sets. In the UCR field trial, entry ratios for both bifenthrin/ zeta-cypermethrin and *Beauveria bassiana* were lower than the control but were not significant ($P > 0.05$) (Fig. 8A). However, gallery success was significantly lower ($P<0.05$) in bifenthrin/ zeta-

cypermethrin compared to all other groups including the control (Fig. 8B). At the CPP site, bifenthrin/ zeta-cypermethrin had the lowest entry ratio compared to all other groups (Fig. 8A). Measuring gallery success, bifenthrin/ zeta-cypermethrin was the only treatment significantly lower (P <0.05) than the control (Fig. 8B). Gallery success in azoxystrobin treatments were lower than the control but this interaction was not significant (P > 0.05).

DISCUSSION

Effective management strategies for commercial avocado are limited for SHB (Eatough-Jones et al. 2015; Mendel et al. 2017) however management in other invasive ambrosia beetle systems has been explored (Carrillo et al. 2015; Mayfield et al. 2008; Peña et al 2011; Ploetz et al 2011; Ploetz et al. 2017). Current management suggestions in other hosts (Eatough-Jones et al. 2015) as well as avocado (Mendel et al. 2017) have suggested removal of infested material to reduce localized populations. In avocado, however, when infested branches are pruned away, beetles may still remain in areas that cannot be pruned away. The best approach is to manage this is a combination of cultural practices such as pruning, removing infested material coupled with application of chemical control strategies, when necessary, to treat remaining populations of SHB that cannot be pruned away. This investigation into pesticides for use in avocado indicate some candidates with significant effectiveness against PSHB and their fungal symbionts from *in vitro* and in field trials. However, the most effective treatment was found to be the contact insecticide bifenthrin/ zeta-cypermethrin. The management strategy of pruning and contact insecticide integrated management can aid in reducing local PSHB population within

avocado groves and help reduce disease pressure and prevent further spread to other areas.

The premix insecticide bifenthrin/zeta-cypermethrin used in this study was the most effective treatment in significantly reducing PSHB gallery initiation and often resulted in insect mortality twenty-four hours post application when used at the maximum label rate. Use of pyrethroid insecticides have been found to be effective against other ambrosia beetles in oak (Svihra et al. 2004), various nursery trees (Reding et al. 2011), and also in avocado in Florida (Peña et al. 2011). Additionally, the use of bifenthrin resulted in a reduction of PSHB attacks in California sycamore (Eatough-Jones 2017). Despite the efficacy of pyrethroid insecticides for the management of several ambrosia beetles including PSHB, the discontinuation of the Section 18 for bifenthrin/ zeta-cypermethrin for PSHB on avocado no longer permits the use of this product and stresses the immediate need for continued research on pyrethroid efficacy in managing PSHB and ultimately the registration of these compounds for use in commercial avocado production since they are shown here and in other studies (Eatough-Jones 2017; Mayorquin et al 2018) to be effective against these pests. The timing of pesticide application should be further investigated to develop an integrated management plan to effectively reduce populations of the pest while reducing the volume of chemical pesticide applied. This may be possible by targeting the pests in the colder winter months since their activity has been previously shown to decrease in these times (Lynch et al. 2018; Mayorquin et al. 2018)

An effort of utilize commercially available fungicide azoxystrobin which is already registered to use to control other common diseases (Schaffer et al 2013) didn't show any inhibition to the growth of the fungal symbionts *in vitro*. Because the high application rate achieves such a mild effect, this fungicide may not be the best approach for this pest and also has been reported ineffective against red bay ambrosia beetle (*Xyloborus glabratus*) in Florida (Ploetz et al. 2011). Thiabendazole was found to completely inhibit all SHB fungal symbionts at a rate lower than the label rate and was also effective in reducing beetle to produce gallery successfully (Fig. 5B). Additionally, incorporation of thiabendazole into sawdust media led to lower mean offspring (Fig. 3) and a lower recovery of *F. euwallaceae*, the primary symbiont of PSHB, in female heads, abdomen, and inside gallery walls (Fig 5). In another study thiabendazole was found to inhibit the growth of *R. lauricola*, the causal agent of Laurel wilt disease, *in vitro* (Mayfield et al. 2008) and has been shown to have fungicidal activity in avocado *in planta* in Florida (Ploetz et al. 2011). However, when thiabendazole was injected into PSHB infested California Sycamore, it was not shown a significantly beetle attack reduction, but a longer retention rate was observed compared to other treatments tested (Mayorquin et al. 2018). There is also evidence of retention from earlier studies utilizing thiabendazole for the treatment of Dutch elm disease (Stennes 1987). The effectiveness of this particular fungicide in the field should be investigated more in the further as a potential treatment against PSHB and KSHB on avocado as well as other hosts.

With the rise in demand for agricultural products generated through organic farming (Ronald and Adamchak 2018), limitations on pesticide use can cause some

difficulty in management decisions of certain foliar plant diseases (Van Bruggen and Finckh 2016). Organic biological control agents such as *B. subtilis* have been of interest because of their anti-fungal properties (Fiddaman and Rossal 1993) and effectiveness against *F. euwallaceae* (Guevara-Avendaño et al. 2018) and *F. kuroshium* (Na 2017). In this study, the maximum label rate for *B. subtilis* was observed to be effective *in vitro* in reducing the mycelial growth of all SHB fungal symbionts as well as when amended in sawdust media. Interestingly, when the commercial product was amended to media with streptomycin and autoclaved, inhibition of all symbiotic fungi and prevention of offspring production from PSHB was achieved indicating the bacteria may be producing heat stable antifungal agent(s), which has been reported previously in *B. subtilis*. (Munimbazi and Bullerman 1998; Walker et al. 1998). For example, a purified novel protein, BTL, from *Bacillus* sp. strain BTL2 from tobacco stems was thermostable, retaining almost 100% activity when heated to 100 °C for 15 min (Zhang et al. 2008). The effective heat stable agent found from strain QST713 in the commercial product should be further investigated since it is clearly effective against PSHB symbionts *in* vitro. *B. subtilis* endophytes from avocado may be better candidates for application onto avocado wood, which have been shown to have similar inhibitory effect to QST713 against *F. euwallaceae* and *F. kuroshium in vitro* and *in planta* experiments on avocado (Na 2016).

The entomopathogen *B. bassiana* used in this study was found to be very effective against PSHB when deposited on sawdust media and moderately effective in bolt and field trials. The reduction in effectiveness on wood application may be because of the lack of optimal conditions for the entomopathogenic fungi to proliferate on the wood as

these treatments were applied toward the end of spring and again in the warm summer months. High humidity in avocado growing regions such as Florida may provide better conditions for this fungus to persist in absence of the target insect host. It has been reported that conidia of *B. bassiana* can be sensitive to other environmental factors such as solar radiation which was greatly improved when applied with 10% humic acid (Kaiser et al. 2018). Biological control applications and descriptions of new candidates should continue to be investigated to find conditions and/or candidates that can increase persistence and effectiveness on treated areas in varying environmental conditions.

High density avocado groves can result in little light penetration into the lower canopy which can be relieved through cultural management practices such as pruning (Bender 2012). The cultural management practice for PSHB infestations involves removal of infested wood and subsequent solarization or chipping of wood pieces to reduce pest pressure from beetle populations (Eatough-Jones et al. 2015). Phytosanitation in avocado has already been recommended for management of PSHB in avocado (Mendel et al. 2017) and can be practiced along with chemical pesticide applications as part of an IPM strategy. Other ambrosia beetles in Taiwan were reported to attack avocado trees in poor health (Hung and Jong 1995) and suggested that maintaining good tree health by good agronomic practice may help reduce attacks, which for organic growers at this point in time, is the best option along with cultural management through pruning until biological control applications methods are innovated.

LITERATURE CITED

Ahimou, F., Jacques, P. and Deleu, M., 2000. Surfactin and iturin A effects on Bacillus subtilis surface hydrophobicity. Enzyme and Microbial Technology, 27(10), 749-754.

Beaver, R.A., 1989. Insect-fungus relationships in the bark and ambrosia beetles., in: Insect-Fungus Interactions. 14th Symposium of the Royal Entomological Society of London in Collaboration with the British Mycological Society. Presented at the 14th Symposium of the Royal Entomological Society of London in collaboration with the British Mycological Society., Academic Press, 121–143.

Bender, Gary. S., Pruning/Canopy Management. Avocado Production in California A Cultural Handbook for Growers, 2nd ed., University of California Coorperative Extension, 2012, 185–198.

Berry D, Selby RD, Horvath JC, Cameron RH, Porqueras D, Stouthamer R (2016) A modular system of 3D printed traps for studying the biology of shot hole borers and other Scolytinae. Journal of Economic Entomology

California Avocado Commission. 2018. Industry Statistical Data, 2016-2017. http://www.californiaavocadogrowers.com/industry/industry-statistical-data.

Carrillo, D., Crane, J.H., Peña, J.E., 2013. Potential of Contact Insecticides to Control Xyleborus glabratus (Coleoptera: Curculionidae), A Vector of Laurel Wilt Disease in Avocados. J. Econ. Entomol. 106, 2286–2295.

Carrillo, D., Narvaez, T., Cossé, A.A., Stouthamer, R. and Cooperband, M., 2015. Attraction of Euwallacea nr. fornicatus (Coleoptera: Curculionidae: Scolytinae) to lures containing quercivorol. Florida Entomologist, 98(2), 780-782.

Castrillo, L.A., Griggs, M.H., Ranger, C.M., Reding, M.E. and Vandenberg, J.D., 2011. Virulence of commercial strains of Beauveria bassiana and Metarhizium brunneum (Ascomycota: Hypocreales) against adult Xylosandrus germanus (Coleoptera: Curculionidae) and impact on brood. Biological Control, 58(2), 121-126.

Cooperband, M.F., Stouthamer, R., Carrillo, D., Eskalen, A., Thibault, T., Cossé, A.A., Castrillo, L.A., Vandenberg, J.D., Rugman-Jones, P.F., 2016. Biology of two members of the Euwallacea fornicatus species complex (Coleoptera: Curculionidae: Scolytinae), recently invasive in the U.S.A., reared on an ambrosia beetle artificial diet. Agr. Forest Entomol. 18, 223–237.

Eatough-Jones, M., Paine, T.D., 2015. Effect of Chipping and Solarization on Emergence and Boring Activity of a Recently Introduced Ambrosia Beetle (Euwallacea sp., Coleoptera: Curculionidae: Scolytinae) in Southern California. J. Econ. Entomol. 108, 1852–1559.

Jones, M.E. and Paine, T.D., 2018. Potential pesticides for control of a recently introduced ambrosia beetle (Euwallacea sp.) in southern California. Journal of pest science, 91(1), 237-246.

Eatough-Jones, M., Kabashima, J., Eskalen, A., Dimson, M., Mayorquin, J.S., Carrillo, J.D., Hanlon, C.C., Paine, T.D., 2017. Evaluations of Insecticides and Fungicides for Reducing Attack Rates of a new invasive ambrosia beetle (Euwallacea Sp., Coleoptera: Curculionidae: Scolytinae) in Infested Landscape Trees in California. J. Econ. Entomol. 110, 1611–1618.

Eskalen, A., Gonzalez, A., Wang, D.H., Twizeyimana, M., Mayorquin, J.S., Lynch, S.C., 2012. First Report of a Fusarium sp. and Its Vector Tea Shot Hole Borer (Euwallacea fornicatus) Causing Fusarium Dieback on Avocado in California. Plant Dis. 96, 1070–1070.

Eskalen, A., Stouthamer, R., Lynch, S.C., Rugman-Jones, P.F., Twizeyimana, M., Gonzalez, A., Thibault, T., 2013. Host Range of Fusarium Dieback and Its Ambrosia Beetle (Coleoptera: Scolytinae) Vector in Southern California. Plant Dis. 97, 938–951.

Fiddaman, P.J. and Rossall, S., 1993. The production of antifungal volatiles by Bacillus subtilis. Journal of Applied Bacteriology, 74(2), 119-126.

Freeman, S., Sharon, M., Maymon, M., Mendel, Z., Protasov, A., Aoki, T., Eskalen, A., O'Donnell, K., 2013. Fusarium euwallaceae sp. nov., a symbiotic fungus of Euwallacea sp., an invasive ambrosia beetle in Israel and California. Mycologia, 105, 1595–1606.

Freeman, S., Sharon, M., Dori-Bachash, M., Maymon, M., Belausov, E., Maoz, Y., Margalit, O., Protasov, A., Mendel, Z., 2016. Symbiotic association of three fungal species throughout the life cycle of the ambrosia beetle Euwallacea nr. fornicatus. Symbiosis, 68, 115–128.

Guevara-Avendaño, E., Carrillo, J.D., Ndinga-Muniania, C., Moreno, K., Méndez-Bravo, A., Guerrero-Analco, J.A., Eskalen, A. and Reverchon, F., 2018. Antifungal activity of avocado rhizobacteria against Fusarium euwallaceae and Graphium spp., associated with Euwallacea spp. nr. fornicatus, and Phytophthora cinnamomi. Antonie van Leeuwenhoek, 111(4), 563-572.

Hothorn, T., Bretz, F. and Westfall, P. 2008. Simultaneous inference in general parametric models. Biometrical J., 50(3), 346-363.

Hulcr, J. and Stelinski, L.L., 2017. The ambrosia symbiosis: from evolutionary ecology to practical management. Annual review of entomology, 62, 285-303.

Hung, S.C. and Jong, T.M.1995.Ambrosia beetle- a weakened avocado tree pest. Shinung Farm magazine 320, 70-71.

Jansson, R.K., Brown, R., Cartwright, B., Cox, D., Dunbar, D.M., Dybas, R.A., Eckel, C., Lasota, J.A., Mookerjee, P.K., Norton, J.A. and Peterson, R.F., 1997. Emamectin benzoate: a novel avermectin derivative for control of lepidopterous pests. Proceedings of the 3rd International Workshop on Management of Diamondback Moth and Other Crucifer Pests. MARDI, Kuala Lumpur, Malaysia.

Johnson, R.M., Ellis, M.D., Mullin, C.A. and Frazier, M., 2010. Pesticides and honey bee toxicity–USA. Apidologie, 41(3), 312-331.

Kaiser, D., Bacher, S., Mène-Saffrané, L. and Grabenweger, G., 2018. Efficiency of natural substances to protect Beauveria bassiana conidia from UV radiation. Pest management science.

Leelasuphakul, W., Sivanunsakul, P. and Phongpaichit, S., 2006. Purification, characterization and synergistic activity of β-1, 3-glucanase and antibiotic extract from an antagonistic Bacillus subtilis NSRS 89-24 against rice blast and sheath blight. Enzyme and Microbial Technology, 38(7), 990-997.

Leifert, C., Li, H., Chidburee, S., Hampson, S., Workman, S., Sigee, D., Epton, H.A.S. and Harbour, A., 1995. Antibiotic production and biocontrol activity by Bacillus subtilis CL27 and Bacillus pumilus CL45. Journal of applied bacteriology, 78(2), 97-108.

Lenth, R., Love, J. and Hervé, M., 2017. Package 'emmeans'. Underst Stat, 34, 216-21.

Lynch, S.C., Twizeyimana, M., Mayorquin, J.S., Wang, D.H., Na, F., Kayim, M., Kasson, M.T., Thu, P.Q., Bateman, C., Rugman-Jones, P. and Hulcr, J., 2016. Identification, pathogenicity and abundance of Paracremonium pembeum sp. nov. and Graphium euwallaceae sp. nov. two newly discovered mycangial associates of the polyphagous shot hole borer (Euwallacea sp.) in California. Mycologia, 108(2), 313-329.

Lynch, S.C., Carrillo, J.D., Stouthamer, R., Eskalen, A. 2018. Severity of Fusarium Dieback – Shot Hole Borers Analyzed. From The Grove. Vol.8, No 4. 46-50

Marrone, P.G., 1999. Microbial pesticides and natural products as alternatives. Outlook on Agriculture, 28(3), 149-154.

Mayfield III, A.E., Barnard, E.L., Smith, J.A., Bernick, S.C., Eickwort, J.M. and Dreaden, T.J., 2008. Effect of propiconazole on laurel wilt disease development in redbay trees and on the pathogen in vitro. Arboriculture & Urban Forestry, 34(5), 317-324.

Mayorquin, J.S., Carrillo, J.D., Twizeyimana, M., Peacock, B.B., Sugino, K.Y., Na, F., Wang, D.H., Kabashima, J.N. and Eskalen, A., 2018. Chemical Management of Invasive Shot Hole Borer and Fusarium Dieback in California Sycamore (Platanus racemosa) in Southern California. Plant Dis. 102(7): 1307-1315.

Mendel, Z., Protasov, A., Sharon, M., Zveibil, A., Yehuda, S.B., O'Donnell, K., Rabaglia, R., Wysoki, M., Freeman, S., 2012. An Asian ambrosia beetle Euwallacea fornicatus and its novel symbiotic fungus Fusarium sp. pose a serious threat to the Israeli avocado industry. Phytoparasitica 40, 235–238.

Mendel, Z., Protasov, A., Maoz, Y., Maymon, M., Miller, G., Elazar, M. and Freeman, S., 2017. The role of Euwallacea nr. fornicatus (Coleoptera: Scolytinae) in the wilt syndrome of avocado trees in Israel. Phytoparasitica, 45(3), 341-359.

Munimbazi, C. and Bullerman, L.B., 1998. Isolation and partial characterization of antifungal metabolites of Bacillus pumilus. Journal of Applied Microbiology, 84(6), 959-968.

Na, F., 2016. 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 (Thesis, UC Riverside).

Na, F., Carrillo, J.D., Mayorquin, J.S., Ndinga-Muniania, C., Stajich, J.E., Stouthamer, R., Huang, Y.T., Lin, Y.T., Chen, C.Y. and Eskalen, A., 2018. Two novel fungal symbionts Fusarium kuroshium sp. nov. and Graphium kuroshium sp. nov. of Kuroshio shot hole borer (Euwallacea sp. nr. fornicatus) cause Fusarium dieback on woody host species in California. *Plant Disease*, PDIS-07.

Norris, D. M. and H. M. Chu (1985) *Xyleborus ferrugineus*. In Hand Book of Insect Rearing. Vol. I (P. Singh and R. Moore, eds.). Elsevier, Amsterdam, 303–315

OC Parks 2016. Shot Hole Borer: Managing the Invasive Beetle. https://oc-parks-gis.maps.arcgis.com/apps/Cascade/index.html?appid=680fd0c9e73f4857a8477791f7ee796f.

Paap, T., De Beer, Z.W., Migliorini, D., Nel, W.J. and Wingfield, M.J., 2018. The polyphagous shot hole borer (PSHB) and its fungal symbiont Fusarium euwallaceae: a new invasion in South Africa. Australasian Plant Pathology, 47(2), 231-237.

Peña, J.E., Crane, J.H., Capinera, J.L., Duncan, R.E., Kendra, P.E., Ploetz, R.C., McLean, S., Brar, G., Thomas, M.C., Cave, R.D., 2011. Chemical Control of the Redbay Ambrosia Beetle, Xyleborus glabratus, and Other Scolytinae (Coleoptera: Curculionidae). Fla. Entomol. 94, 882–896.

Ploetz, R.C., Pérez-Martínez, J.M., Evans, E.A., Inch, S.A., 2011. Toward Fungicidal Management of Laurel Wilt of Avocado. Plant Dis. 95, 977–982.

Ploetz, R. C., Hughes, M. A., Kendra, P. E., Fraedrich, S. W., Carrillo, D., Stelinski, L. L., Hulcr, J., Mayfield, A. E., III, Dreaden, T. L., Crane, J. H., Evans, E. A., Schaffer, B. A., and Rollins, J. A. 2017. Recovery plan for laurel wilt of avocado, caused by Raffaelea lauricola. Plant Health Prog. 18:51-77.

Reding, M.E. and Ranger, C.M., 2011. Systemic insecticides reduce feeding, survival, and fecundity of adult black vine weevils (Coleoptera: Curculionidae) on a variety of ornamental nursery crops. Journal of economic entomology, 104(2), 405-413.

Ronald, P.C. and Adamchak, R.W., 2018. Tomorrow's table: organic farming, genetics, and the future of food. Oxford University Press.

Sarkar, D. 2008. Lattice: multivariate data visualization with R. Springer Science & Business Media.

Schaffer, B.A., Wolstenholme, B.N. and Whiley, A.W. eds., 2013. The avocado: botany, production and uses. CABI.

Selvasundaram, R. and Muraleedharan, N., 2000. Occurrence of the entomogenous fungus Beauveria bassiana on the shot hole borer of tea. Journal of Plantation Crops, 28(3), 229-230.

Slawecki, R.A., Ryan, E.P. and Young, D.H., 2002. Novel fungitoxicity assays for inhibition of germination-associated adhesion of Botrytis cinerea and Puccinia recondita spores. Applied and environmental microbiology, 68(2), 597-601.

Stennes, M.A., 1987. Distribution and Retention of Thiabendazole Hypophosphite and Carbendazim Phosphate Injected into Mature American Elms. Phytopathology 77, 707–712.

Stouthamer, R., Rugman-Jones, P., Thu, P.Q., Eskalen, A., Thibault, T., Hulcr, J., Wang, L.J., Jordal, B.H., Chen, C.Y., Cooperband, M. and Lin, C.S., 2017. Tracing the origin of a cryptic invader: phylogeography of the E uwallacea fornicatus (C oleoptera: C urculionidae: S colytinae) species complex. Agricultural and forest entomology, 19(4), 366-375.

Svihra, P., Crosby, D.F. and Duckles, B., 2004. Emergence suppression of bark and ambrosia beetles in infested oaks. Journal of Arboriculture. 30: 62-66

U.S. Department of Agriculture. New Pest Response Guidelines: Exotic Wood-Boring and Bark Beetles. Anonymous. Available at: https://www.aphis.usda.gov/import_export/plants/manuals/emergency/downloads/nprg-wood_boring_bark_beetles.pdf. Accessed: 4.7.17

Van Bruggen, A.H.C. and Finckh, M.R., 2016. Plant diseases and management approaches in organic farming systems. Annual review of phytopathology, 54, 25-54.

Venables, W.N. and Ripley, B.D., 2002. Tree-based methods. Modern Applied Statistics with S (251-269). Springer, New York, NY.

Walker, R., Powell, A.A. and Seddon, B., 1998. Bacillus isolates from the spermosphere of peas and dwarf French beans with antifungal activity against Botrytis cinerea and Pythium species. Journal of Applied Microbiology, 84(5), 791-801.

Wickham, H., 2016. ggplot2: elegant graphics for data analysis. Springer.

Zhang, B., Xie, C. and Yang, X., 2008. A novel small antifungal peptide from Bacillus strain B-TL2 isolated from tobacco stems. Peptides, 29(3), 350-355.

Table 4.1. Pesticides used in this study

Active ingredient	Chemical family	Trade Name	Manufacturer	Rate(s) Applied[a]
Bacillus subtilis	Biological	Serenade ASO	Bayer	30% (v/v) solution
Beauvaria bassiana	Biological	Mycotrol WPO	BioWorks	$9x10^{10}$ spores/ml
Azoxystrobin	Strobilurin	Abound	Syngenta	2700 ppm
Thiabendazole	Benzimidazole	Arbotect-20S	Syngenta	50 ppm
Bifenthrin/Zeta-Cypermethrin	Pyrethroid	Hero	FMC	262, 100, 50, 25, 10 ppm

[a] Multiple rates were only used for Bifenthrin/Zeta-Cypermethrin in one portion of the study

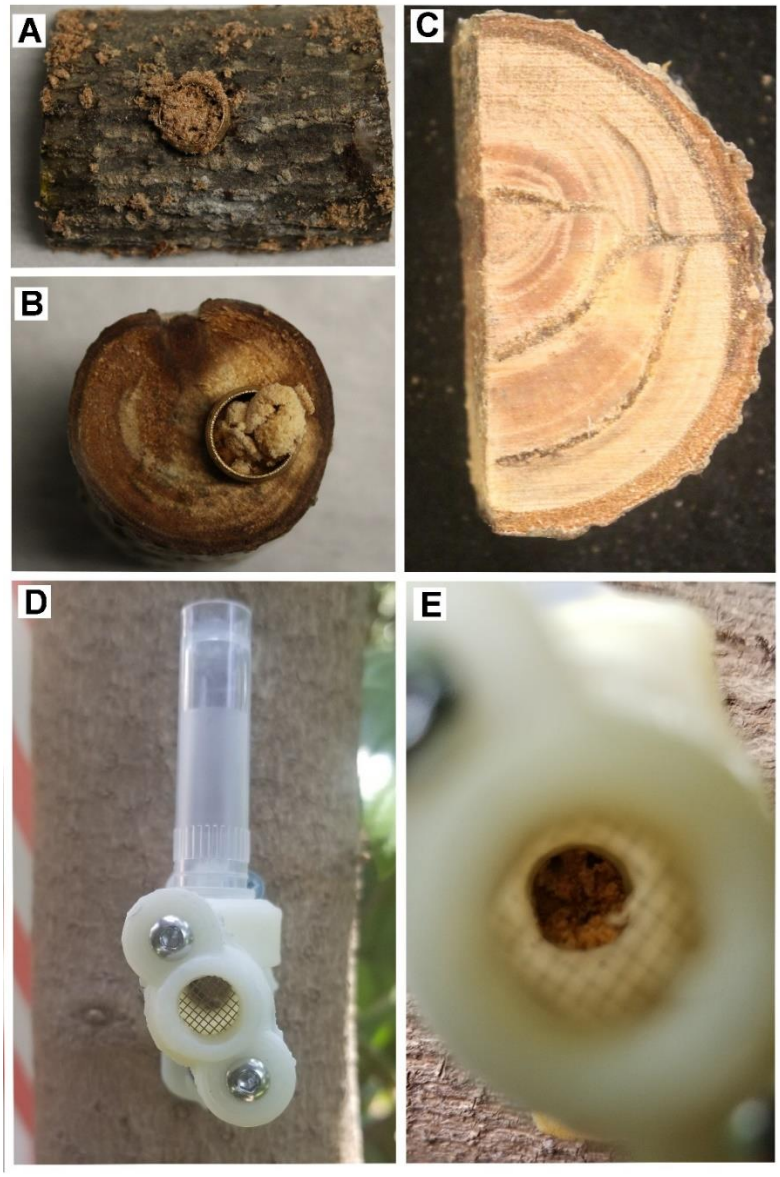

Figure 4.1. Visible frass from the “bark” type cut non-treated avocado bolt (A). Visible frass from the “prune” type cut non-treated avocado bolt (B). “Bark” type cut cross section from a bandsaw pass along the entry hole representing “gallery success” (C). 3D printed traps inverted after beetle introduction with four inseminated females (D). Visible frass generated from entry of the introduced females into avocado branches (E).

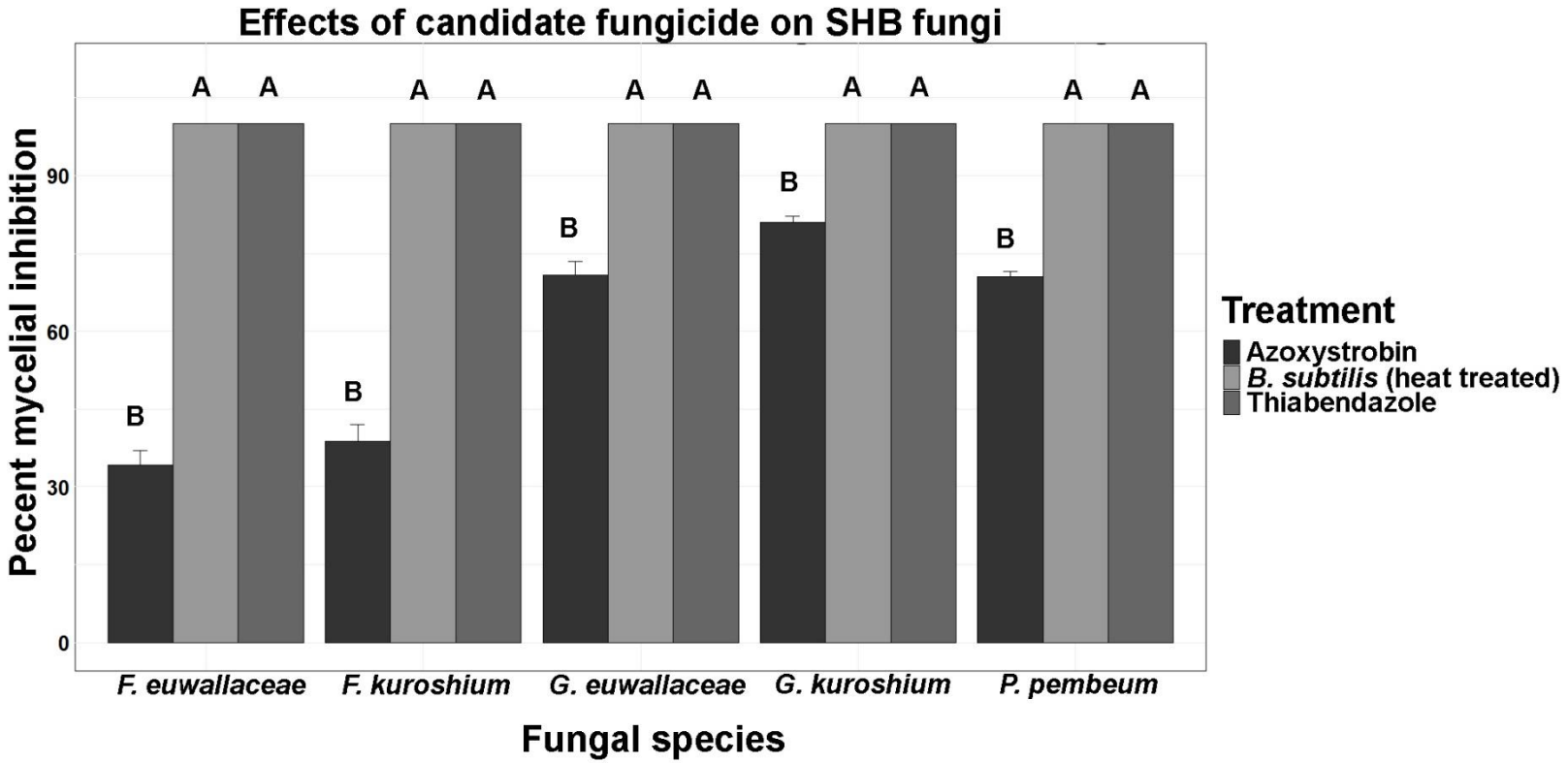

Figure 4.2. Percent mycelial inhibition on SHB fungal symbionts from media amended with fungicide treatments. Letters indicate significance from EMM analysis at α=0.05 within each species.

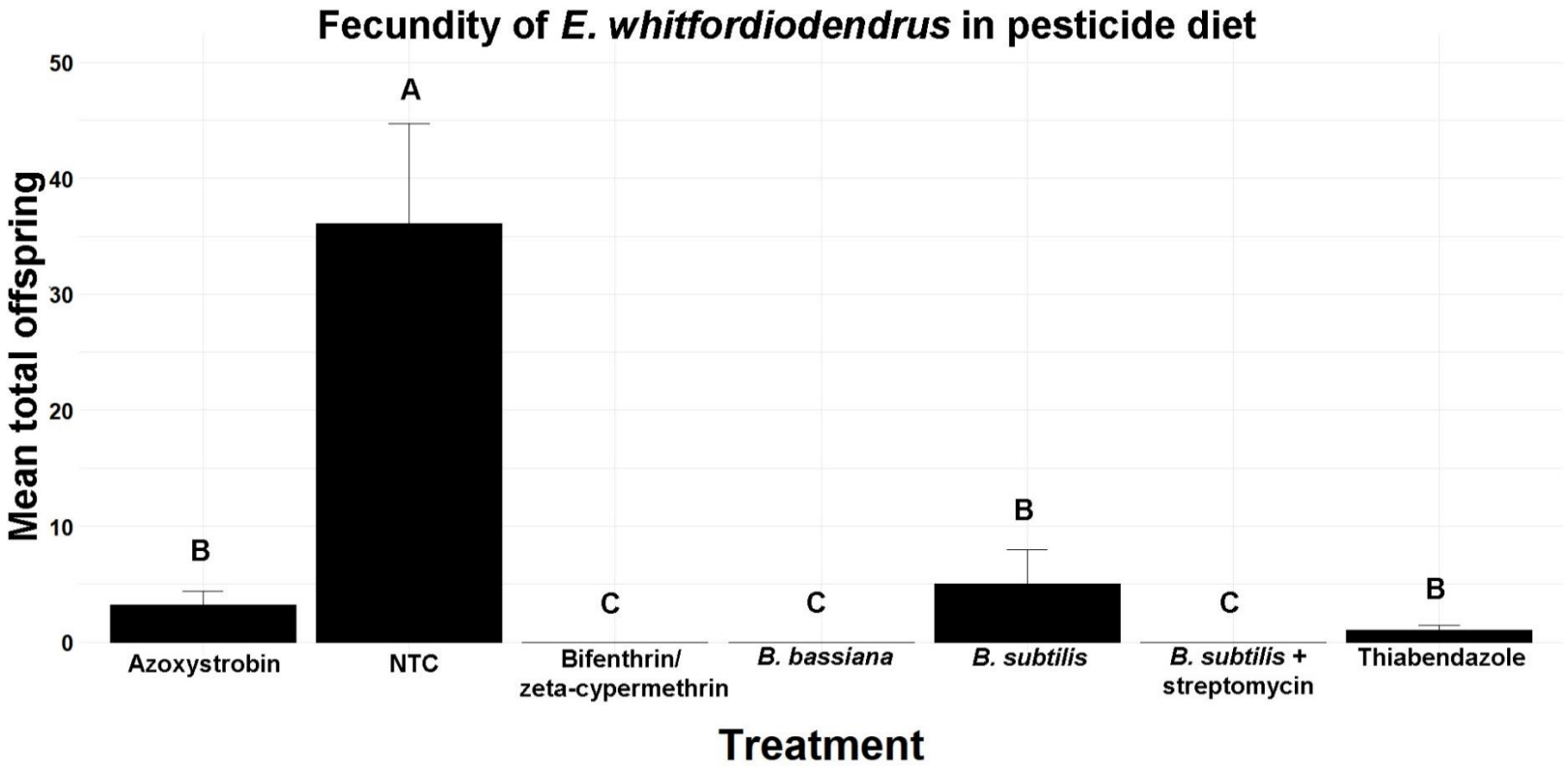

Figure 4.3. Total offspring from sawdust media amended with pesticide treatments with NTC representing the non-treated control. Letters indicate significance from EMMAnalysis at $\alpha=0.05$ comparing treatments to each other.

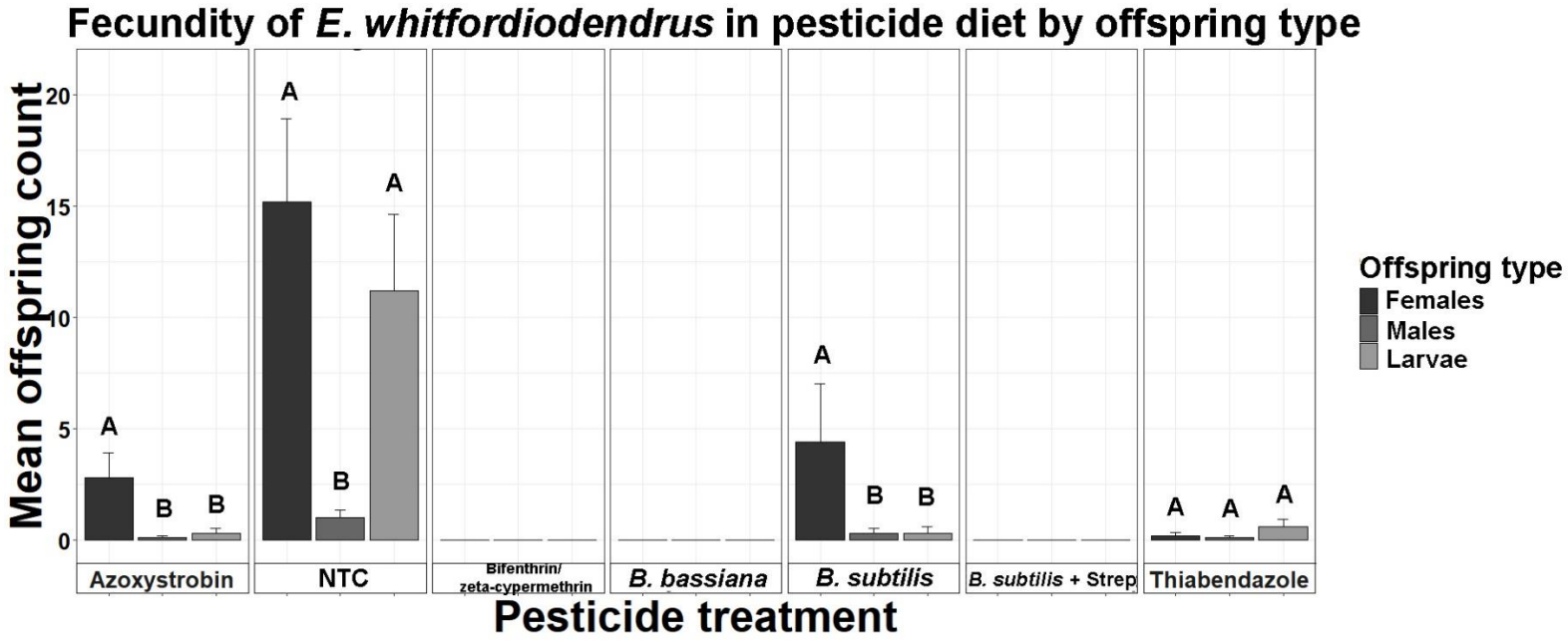

Figure 4.4. Counts of offspring from different sample types from sawdust media amended with pesticide treatments. Letters indicate significance from EMM analysis at α=0.05 comparing offspring types to each other within each treatment.

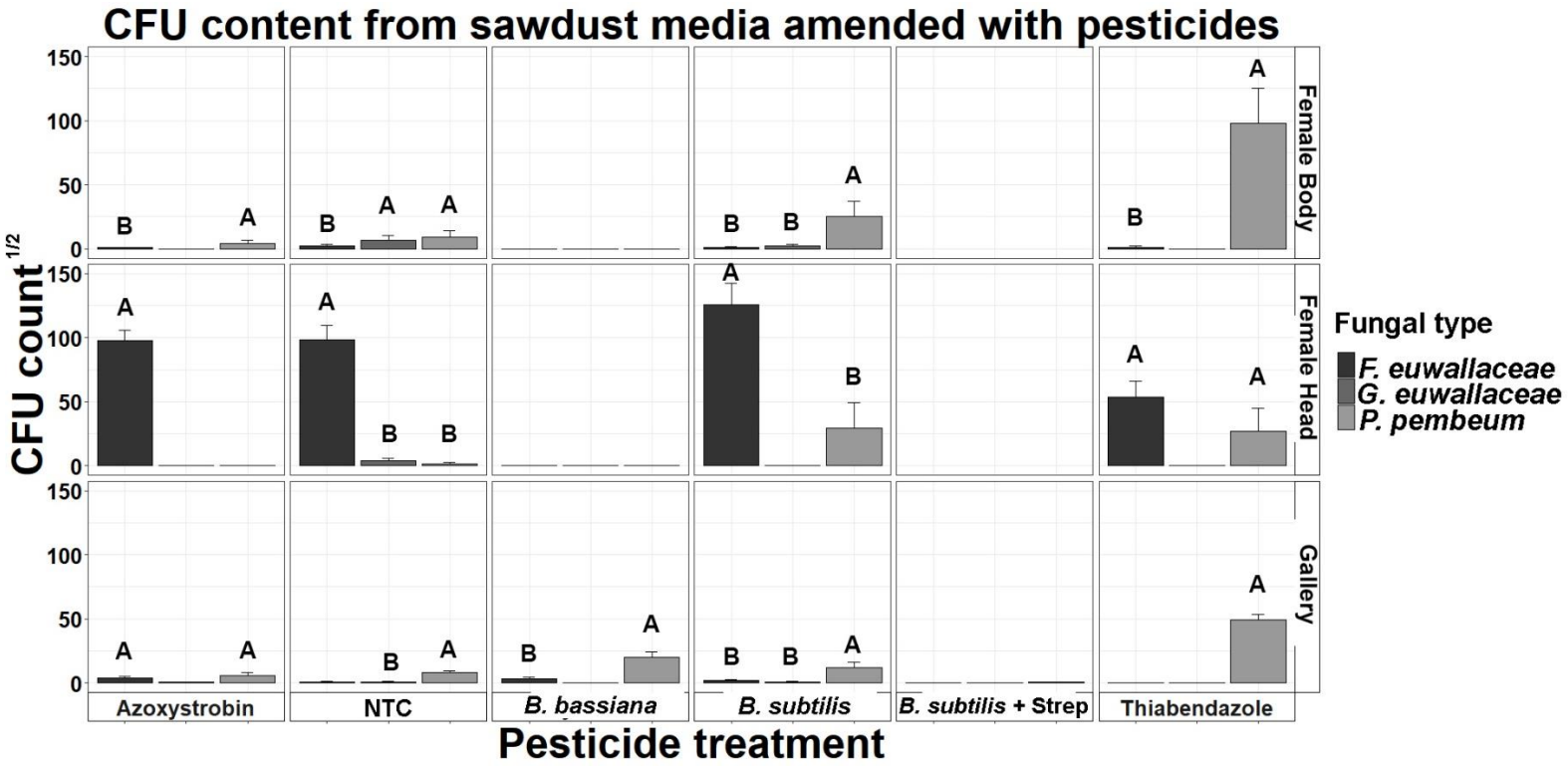

Figure 4.5. CFU content recovered from female head (mycangia), bodies (guts), and gallery samples from sawdust media amended with pesticide treatments. CFU values were transformed by square root and letters indicate significance from EMM analysis at α=0.05 comparing fungal species to each other within each facet

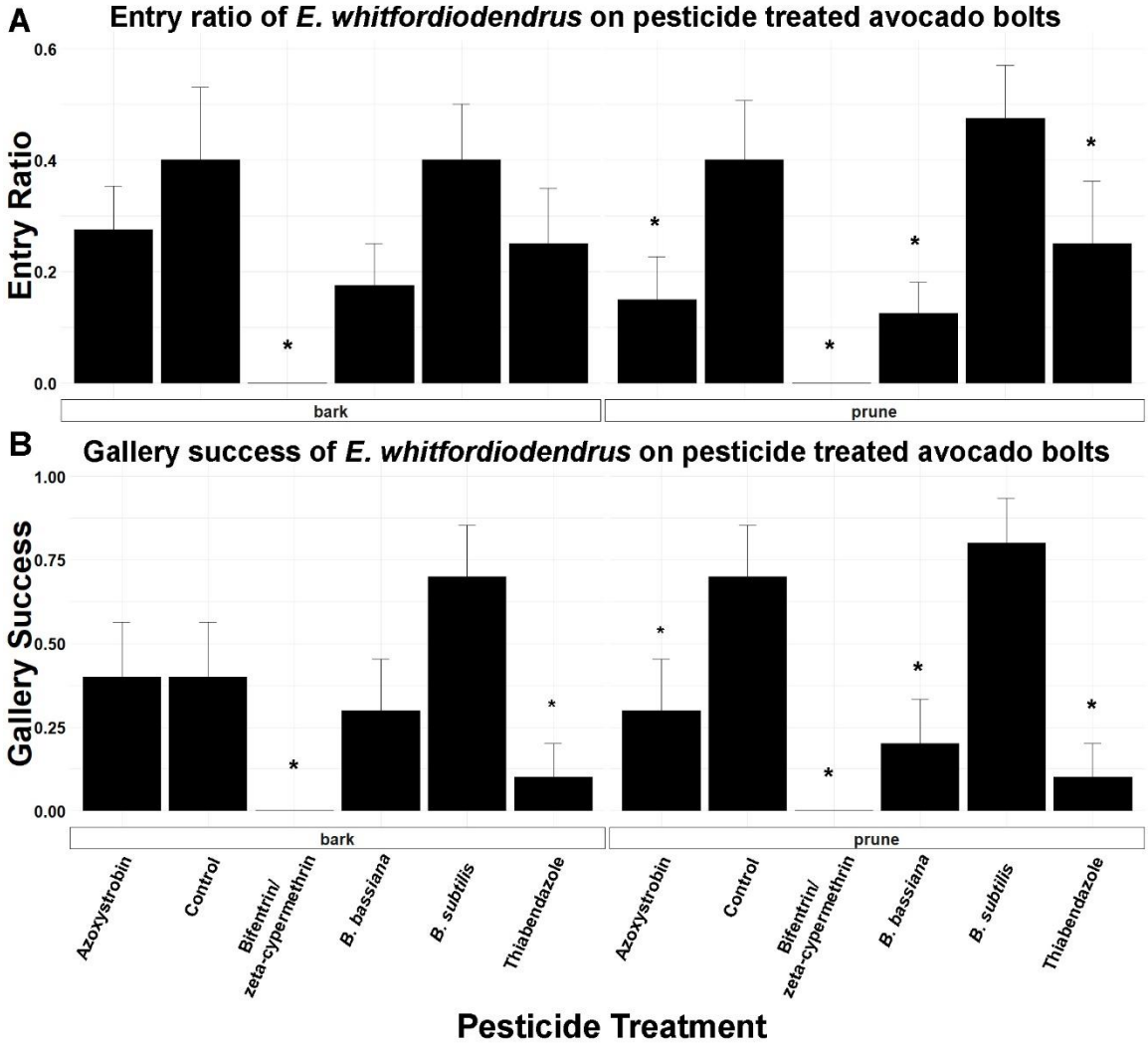

Figure 4.6. Entry ratios of PSHB into avocado bolts spray treated with pesticides from both "bark" and "prune" type cuts (A). Gallery success of PSHB into avocado bolts spray treated with pesticides from both "bark" and "prune" type cuts (B). Asterisks indicate significance from EMM analysis at α=0.05 comparing fungal species to the non-treated control (NTC) within each bolt type

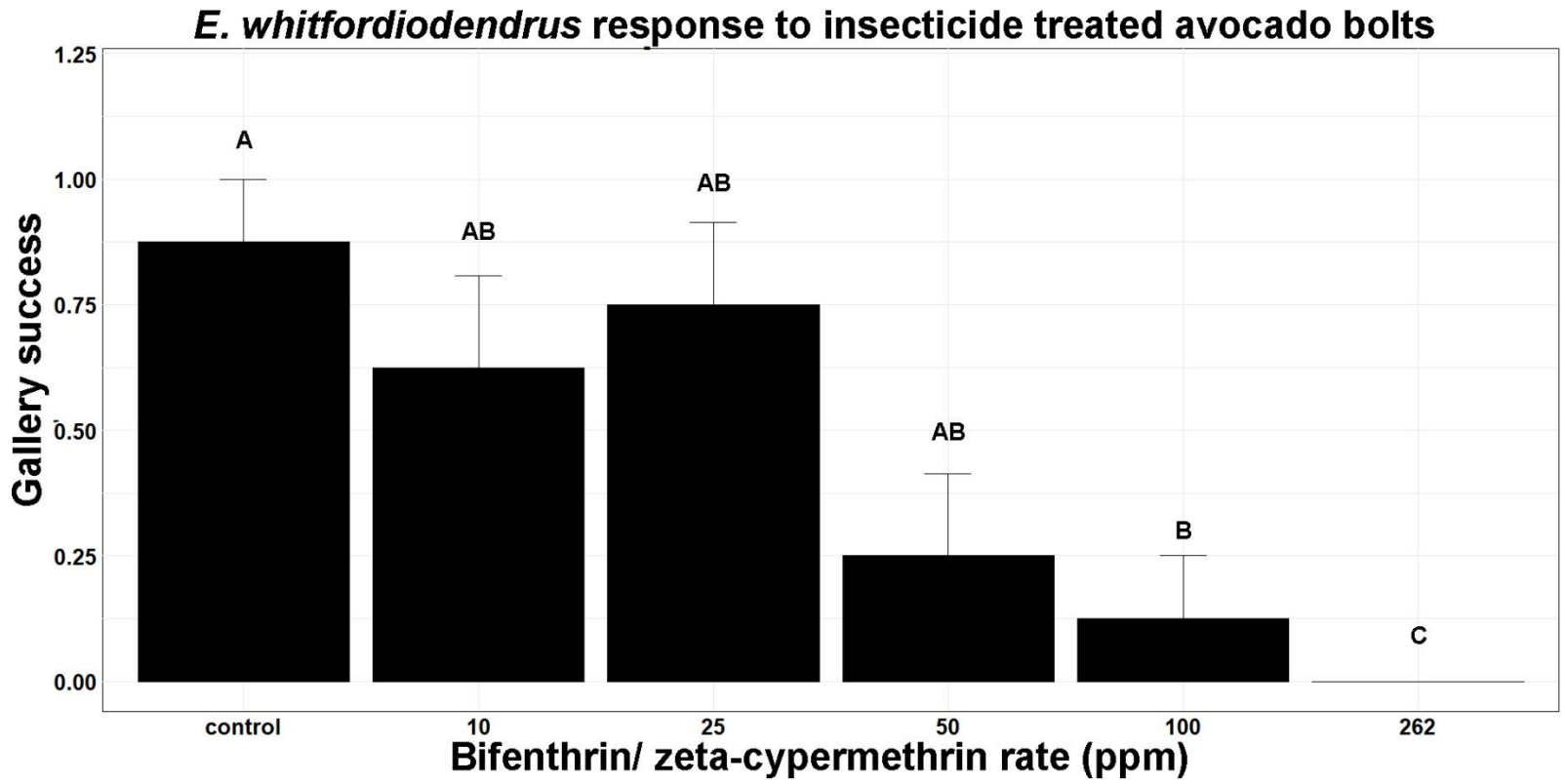

Figure 4.7. Gallery success of PSHB in avocado bolts five days post treatment with different rates of bifentrin/zeta cypermethrin. Letters indicate significance from EMM analysis at α=0.05 comparing applied treatment rates to each other.

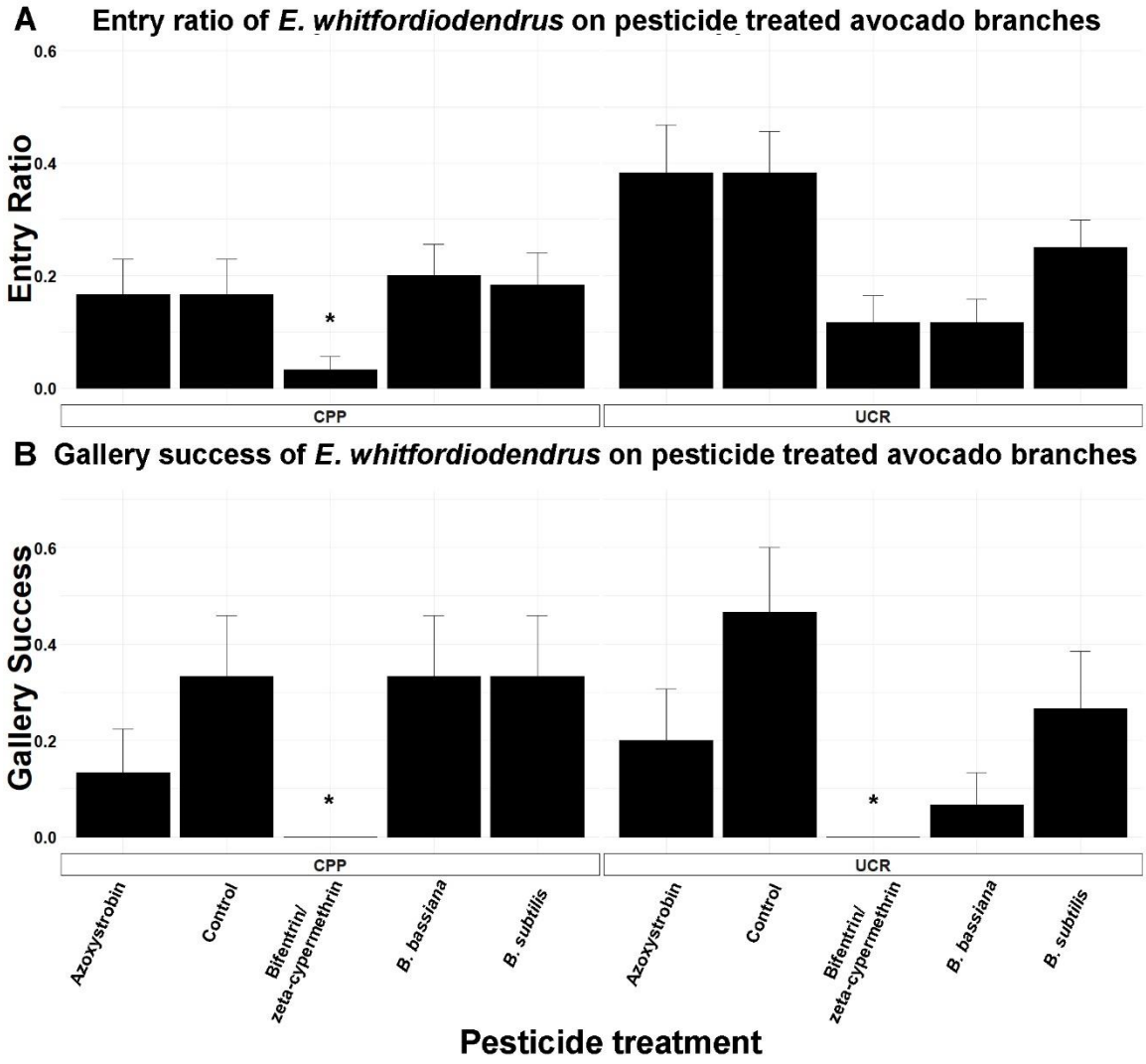

Figure 4.8. Entry ratios for PSHB introduced via 3D traps in pesticide treated avocado wood recovered from field trials (A). Gallery success for PSHB introduced via 3D traps in pesticide treated avocado wood recovered from field trials (B). Asterisks indicate significance from EMM analysis at α=0.05 comparing fungal species to the non-treated control (NTC) within each plot.

GENERAL CONCLUSION

The invasive shot hole borers affecting California landscapes are a continued topic of concern for the region and the health of urban landscapes, native habitats, and the agricultural commodity avocado. The lack of a reliable and rapid diagnostic method for detection of the pathogens involved in the pest disease complexes in California was previously difficult to diagnose in absence of a beetle to sample at the point of attack. This work provides a reliable rapid diagnostic detection method based on two multiplexed real-time qPCR assays which can detect *all* described fungal pathogens vectored by *E. whitfordiodendrus* and *E. kuroshio*, respectively, in California from sample types including fungal cultures, avocado wood, as well as female heads (mycangia). The assays were shown to be sensitive for all targets and are able to qualitatively identify the pathogens from a sample, and quantify within samples as well. The use of such methods provides land managers, arborists, and avocado growers with a reliable diagnostic tool to detect these pests from suspect attacks in early stages of FD as well as track disease progress in California, which can ultimately aid in prompt management decisions for this pest.

The relationship between *Euwallacea* spp. and their symbiotic ambrosia fungi in native regions such as Taiwan were determined to be a promiscuous mutualism as opposed to exclusive. *E. whitfordiodendrus* and *E. fornicatus* were found to vector similar fungal species, which belonged to the *Fusarium* (AFC)*, Graphium,* and *Paracremonium* genera, with some new symbiotic ambrosia fungi species identified within AFC clade B. Members within the AFC were determined to be heterothallic with

both mating types of *Fusarium* spp. detected in 86% of the seven detected phylogenetic species clades. Both mating types (*MAT1-1-1* and *MAT1-2-1*) were recovered from *E. whitfordiodendrus* and *E. fornicatus* indicating they are providing opportunities for fungal-fungal interactions and possibly sexual recombination through their vectoring of the AFC members. However, it is still unknown if mating can occur in fungi involved with *Euwallacea* spp. and what effects it can have on their symbiosis as well as the effects on pathogenicity in the pest disease complexes, which should be investigated in future studies. The novel mating type PCR assays designed in this work to target AFC Fusaria will provide a valuable diagnostic tool to type invasive AFC members in regions other than California which appears to only contain one mating type (*MAT1-1-1*) from *F. euwallaceae* and *F. kuroshium.*

The observed nature of *Euwallacea* spp. with their fungi in Taiwan indicated the relationship between *E. whitfordiodendrus* and *E. fornicatus* and their symbiotic ambrosia fungi may be less exclusive than previously thought. Artificial diet studies determined that *E. whitfordiodendrus* and *E. fornicatus* can survive on each other's symbiotic fungi with no significant difference from feeding on their own, previously thought exclusive, symbiotic ambrosia fungi. Both beetles and their fungi are currently confirmed present in Orange and San Diego Counties which may present opportunities for both beetles to co-exist on the same host and obtain new fungal symbionts through fungal sharing or stealing in close proximity. The effects of this potential exchange are unknown, however *F. euwallaceae* and *F. kuroshium* were positively identified on five attacked trees in orange county in this work, indicating the fungal sharing events may be

already occurring. If this is indeed the case, diagnostic assays such as the qPCR assay presented in this work should be used in conjunction with beetle identification assays to confirm this event and the effects on hosts should be investigated.

The present infestation of the pest disease complexes on avocado is a continued concern but provided in this dissertation are some options utilizing registered pesticides and future directions for integrated management of this pest. Of all the pesticide treatments tested, pyrethroid treatments yielded the best results with the emergency registered section 18 bifenthrin/zeta-cypermethrin (Hero®) product the most effective in killing the beetles from spray treatments. Targeting the fungi involved in the symbiosis continues to be a difficult task as the registered azoxystrobin fungicide has little effects on the beetle's ability to establish galleries. The antifungal product of *B. subtilus* and the unregistered fungicide thiabendazole may have some potential but it is clear from this study that treatments targeting the insects were most effective, although more work into controlling both aspects of the symbiosis should continue to be researched. Increased efforts in monitoring not only the grove but surrounding areas with potential hosts can be important in predicting and controlling spread. Although, it is unlikely to completely eliminate the pests from infested groves and nearby areas, prompt management using the options presented in this work can aid in reducing beetle populations and preventing major loss in avocado production. All data and code used in this study is available at https://github.com/jcarr022/Dissertation_Data