UNIVERSITY OF CALIFORNIA
RIVERSIDE

Natural Enemies of the *Euwallacea fornicates* Species Complex (Coleoptera: Scolytinae) and Their Potential in a Classical Biological Control Program

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

Doctor of Philosophy

in

Entomology

by

Deena W Husein

March 2023

Dissertation Committee:
Dr. Richard Stouthamer, Chairperson
Dr. Timothy Paine
Dr. Thomas Perring

The Dissertation of Deena W Husein is approved:

_____

_____

_____

Committee Chairperson

University of California, Riverside

Acknowledgments

I would first like to thank my major professor, Dr. Richard Stouthamer, for accepting me into his lab and providing unyielding support over the course of my doctoral research at UCR. His guidance has allowed me to grow as an independent researcher by looking at the bigger picture when conducting experiments, to ask appropriate scientific questions when approaching new projects, and, most importantly, to always keep it stupid, simple in every shape and form. I would also like to extend my deepest gratitude to my committee members, Dr. Timothy Paine for his overall aid and guidance since the start of my journey as a Ph.D. student, and Dr. Thomas Perring for all the help and valuable feedback you have provided to me. You all have allowed me to accomplish my goals and gain the necessary knowledge and skills to complete my projects. I know I've become a better scientist thanks to you all.

I would like to acknowledge both past and present colleagues in the Stouthamer lab who have made me feel welcomed with their support along with their helpful tips and feedback to improve my experiments. First and foremost, I would love to thank Dr. Paul F. Rugman-Jones for being an above and beyond mentor that has guided and helped me gain applied experience throughout my experiments during my time at here UCR- not to mention our sessions of comedic relief which I will regularly miss; Chrissy Dodge, for being an amazing role model and sharing insightful information to better facilitate my beetle-rearing experiments; Iris Chien for her loving friendship and continuous support with parasitoid and nematode imaging; Jose Alvarado, Taha Farooqie, Valeh Ebrahimi, Jedeliza Ferrater, Francesc Gomez Marco, Marco Gebiola, and Veronica Fernandez for

their assistance and being patient in both the successful and not so successful ideas that popped in my head. I would also like to thank the undergraduate students who have helped and were partially involved in my research: Rattanan Chungsawat and Choi Chow. You all have made my time here much more enjoyable.

The text of this dissertation, in part, is a reprint of the material as it appears in "Parasitoids associated with ambrosia beetles in the *Euwallacea fornicates* species complex in Taiwan (Coleoptera: Curculionidae: Scolytinae)" (in review). The co-authors Ricky Lara, Chloe Fang-Ling, Yi-Chang Liao, and Hui-Hong Liang assisted with material collection in Taiwan. The co-author Iris Chien helped in data collection and imaging. The co-authors Paul Rugman-Jones and Richard Stouthamer helped design and analyze the data. The co-authors Richard Stouthamer and Susan Shu-Jen directed and supervised the research.

This research was supported by the Robert and Peggy van den Bosch Scholarship for Graduate Student Research in Biological Control and the Harry Scott Smith Biological Control Scholarship in addition to USDA-APHIS grants: 16-8130-382-CA, 18PPQ&TOOC097, 19PPQ&TOOC076, funded by USDA Farm Bill Section 10007 and Plant Protection Act 7721.

I would like to extend my sincere thanks to everyone, past and present, from the admissions office that helped facilitate all the paperwork needed to ease my time as a graduate student. In particular, my thanks go out to Bill Sutton, Breanne Juarez, Cynthia Mena, Ashley Hix, Robin Testa, Paige Mejia, Wendi Tapia as well as Kathy Redd. Thank you all for your help and the comedic relief during my spontaneous visits.

I am also extremely grateful to all the faculty members that provided additional mentoring and guidance whenever I needed it. In particular, I thank Dr. Kerry Mauck, Dr. Hollis Woodard, Dr. Christiane Weirauch, Dr. Dong-Hwan Choe, Dr. Jessica Purcell, Dr. Imad Bayoun, Dr. John Heraty, Dr. Roger Burks, Dr. Quinn McFrederick and Dr. Richard Redak. Truthfully, everyone in this department has been amazing. I was able to complete my journey thanks to your guidance, kindness, and unimaginable support.

To the remarkable members of my graduate cohort, Tessa, Mari, Mark, Kaleigh, Stephanie, Krissy, and especially Carlos, thank you for your friendship and support during our time together.

To my crazy family and amazing friends, thank you all for always giving me that extra dose of love and reassurance that made my time at UCR more memorable. In particular, I'm grateful to Michelle, Najla, Nithya, Daniel, Chrissy, Veronica, Jose, and especially Tessa- for being my hype friend and number one enabler. You all believed in me the most in times when I was doubtful of myself.

To my partner, Brendan, your questionable humor and love gave me just the right amount of strength to make consistent progress in both graduate school and my career. Thank you from the bottom of my heart.

Dedication

This dissertation is dedicated to my mother, Hanan Ayesh, for being my rock and supporting me in every way possible during this journey.

ABSTRACT OF THE DISSERTATION

Natural Enemies of the *Euwallacea fornicates* Species Complex (Coleoptera: Scolytinae) and Their Potential in a Classical Biological Control Program

by

Deena W Husein

Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, March 2023
Dr. Richard Stouthamer, Chairperson

The *Euwallacea fornicatus* species complex, collectively known as shothole borers (SHB), is comprised of three major invasive cryptic ambrosia beetles of economic importance. Since their detection, two of these species, the polyphagous shot hole borer (PSHB), *Euwallacea fornicatus*, and the Kuroshio shothole borer (KSHB), *Euwallacea kuroshio*, have become established in southern California without effective and cost-efficient control measures. The goal of this dissertation was to find promising natural enemies associated with the *E. fornicatus* species complex and assess their potential as candidates in a classical biological control program. In chapter 1, I recorded emergence and behavior of parasitoid associated with shothole borer-infested wood material imported from the beetle's native region. As a result, I identified three parasitoid wasps as natural enemies of PSHB that ions emerged from experimental logs selectively infested with PSHB. In Chapter 2, I attempted to confirm a molecular link between beetles in the *E. fornicatus* species complex and each parasitoid species using molecular gut content analysis. A large subset of parasitoid wasps from Taiwan, collected from

shothole borer-infested wood and yellow panel traps, were sequenced and analyzed for trace amounts of DNA from their host beetles. While a few parasitoid species amplified trace amounts of shothole borer DNA, two braconid parasitoids in the subfamily Euphorinae, along with two parasitoids in the families Bethylidae and Eulophidae amplified shothole borer DNA. In chapter 3, I conducted experiments to identify entomophilic nematodes associated with the *E. fornicatus* species complex. In doing so, a nematode species in the genus *Rhabditolaimus* originally detected in Taiwan, the native region to the *E. fornicatus* species complex, was confirmed to already be established in southern California. Fitness cost experiments indicated adverse effects of this *Rhabditolaimus* sp. on PSHB brood size when reared in the presence of the nematode. This could be evidence that this nematode is a cryptic factor behind the decline of shothole borer populations in previously-infested regions across southern California.

Table of Contents

List of Figures

Figure **Page**

List of Tables

Table **Page**

Introduction

Ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae) are a niche group of insects, characterized by their association with symbiotic fungi, that can be of considerable economic importance worldwide (Browne, 1961). While the majority of ambrosia beetles traditionally colonize stressed or recently dead trees in temperate and tropical regions (Hulcr et al., 2007), a minority within the tribe Xyleborini (Scolytinae) attack and colonize healthy host plants (Paine et al., 1997; Kirkendall et al., 2015**).** This includes members of the *Euwallacea fornicatus* species complex. The *E.fornicatus* species complex comprises of at least four cryptic invasive ambrosia beetles of indistinguishable morphological features (Stouthamer et al., 2017; Gomez et al., 2018; Smith et al., 2019**)**. One member, in particular, is the invasive polyphagous shothole borer (PSHB), *Euwallacea fornicatus* Eichhoff, that first was detected in southern California in 2003 (Eskalen et al., 2012). The PSHB beetle initially was identified morphologically as the tea shothole borer (TSHB), *Euwallacea perbrevis* Schedl, a substantial pest of tea in Sri Lanka. This misidentification dissuaded the normal regulatory quarantine response the now-established pest (Rabaglia et al., 2006). A subsequent genetic survey of shothole borer populations in southern California and their native range in Southeast Asia (Stouthamer et al., 2017) not only uncovered the identity of PSHB across southern California, but also revealed that another member of the *E. fornicatus* species complex, the Kuroshio shothole borer (KSHB), *Euwallacea kuroshio* Gomez & Hulcr had invaded southern California. Large, established populations were found in San Diego County, and more recently in Santa Barbara County (Boland, 2016).

Ambrosia beetles are known for their farming of fungi that predominantly in the genera *Ambrosiella*, *Fusarium*, and *Rafaelea* (Batra, 1963; Hulcr and Stelinski, 2017). Ambrosia fungal spores are stored by the beetles, in specialized internal sacs called mycangia, and later cultivated as the main source of nutrition for both the female adult beetle and her offspring (Batra, 1963; Beaver, 1968). Furthermore, all ambrosia beetles have a haplo-diploid reproductive system, in which unfertilized eggs become males (haploid) and fertilized eggs become females (diploid) (Farrell et al., 2001; Jordal et al., 2000). This allows a mother to determine the sex ratio of her offspring, which she does in a manner consistent with local mate competition theory (Hamilton, 1967). Mothers will produce a very female biased sex ratio, with winged females, and males that are unable to fly (Beaver, 1989). Once male and female siblings reach adulthood, brothers typically mate with their sisters, which will then exit their natal galleries and disperse to a new host and eventually repeat the cycle.

Historically members of the *E. fornicatus* species complex have invaded many areas outside their native range (CABI, 2021). The most recent examples of invasion by PSHB include Israel, South Africa, and Australia (Cook and Broughton, 2023), and KSHB has expanded its range south from California into Mexico, highlighting the ability of these beetles to bypass monitoring strategies (Haack, 2003). The damage they cause stems from two aspects of their feeding habits: the construction of galleries and the infection of their plant hosts with fungi. The gallery construction can case stems and branches to break, and damages the water transport ability of the xylem vessels. In addition the fungal growth will also cause clogging of the xylem vessels, ultimately

depriving host plants of access to water and nutrients (Mendel et al.,2012; Eskalen et al., 2012). Over time, severe infestations from beetles in the *E. fornicatus* species complex can lead to the mortality of the host plant within 2-3 years (Eskalen et al., 2012), and have a dramatic impact on natural and agricultural treescapes. As a result, the fungal associates of both PSHB and KSHB have also been identified (Freeman et al., 2013; Na et al., 2018). The most important associates are *Fusarium euwallacea* Freeman, Mendel, Aoki, & O'Donnell for the PSHB, and *Fusarium kuroshium* Na, Carrillo, & Eskalen for the KSHB, and their pathogenicity towards a wide range of host plants has been documented (e.g., Eskalen et al., 2013; Gomez et al., 2019).

The number and diversity of plants that PSHB can attack is extensive. Eskalen et al. (2013) reported over 200 tree species in southern California the beetle can attack with the ability to reproduce on at least 37. These include several native oaks, maples, sycamores, and willows, in addition to ornamental plants and avocado, a major agricultural crop. More recently, that list has been updated to 412 plant species across 75 families, of which 20 new reproductive hosts were added (Gomez et al., 2019). Research by McPherson et al (2017) estimated that 23.2 million trees (32.8%) in southern California were susceptible to attacks by the *E. fornicatus* species complex. Should half of those numbers become infested, the estimated cost of removing and replacing those trees is $15.9 billion, with an annual accruement of $616.9 million over the next 10 years (McPherson et al., 2017). Thus, there is an urgent need to control the spread of these shothole borers.

Chemical control of shothole borers

Conventional control methods are ineffective on dense infestations of scolytines in part due to the beetle's short dispersal time outside the plant host. This renders topical insecticides inefficient, especially once a population of PSHB has become established in an area. Additionally, systemic insecticides pose a similar limitation since the xylem tissue of an infested host plant is compromised due to the burrowing activity of the beetle, and fungal accumulation that inhibit the transport of both water and pesticide. Another alternative solution includes the use of potassium acetate in the fertilizer, which has been found to reduce the number of eggs and pupae. However, this approach did not result in any significant differences in the total number of galleries, which may further enable Fusarium growth and negatively impact the plant host (Wickremasinghe and Thirugnanasuntharan, 1980).

The use of chemical lures to interrupt mating is also somewhat of a lost cause in the *E. fornicatus* species complex. In many scolytine beetles, males and/or females rely on intraspecific communication that is based on the release of aggregation pheromones to attract the opposite sex for mating (Borden, 1989). Pioneer beetles that land on a host tree, females in monogamous species and males in polygamous species, are usually the ones that release these aggregation pheromones (Borden, 1989). However, unlike Scolytine beetles that rely on aggregation/sex pheromones to mate, PSHB and KSHB females have no need for such pheromones since they mate with their male siblings in the galleries prior to dispersing. Thus the deployment of pheromone lures under an 'attract and kill' strategy would not work. Given the limitation to these control strategies,

classical biological control may be a promising solution to suppress populations of the *E. fornicatus* species complex.

Biological control and Scolytinae

A well known biological control program was the population suppression of the cottony cushion scale (*Icerya purchasi*) in 1888–1889 in California by the vedalia beetle (*Rodolia cardinalis* Mulsant) and the tachinid fly (*Chryptochetum iceryae* Williston) that were imported from Australia (DeBach, 1964). Public awareness of this case encouraged the introduction of exotic species, which began a new era of emphasis on classical biological control. In biological control programs, accurate identification of the pests geographical range is critical to determine the locations where natural enemies of the pest may be found.

Based on phylogeographic analyses taken from surveys conducted worldwide, all members of the *E. fornicatus* species complex were found in Taiwan, which further suggests the possibility of finding natural enemies in this native region (Stouthamer et al., 2017). However, the biological control approach with ambrosia beetles faces many challenges compared to monocrop agricultural systems. While the high diversity of species in forest communities is conceptually advantageous for the collection of natural enemies, the pitfalls become apparent as a result of the myriad of multitrophic interactions that take place in forest-dwelling communities. This reduces the probability of finding the host's natural enemy. The cryptic lifestyle of all ambrosia beetles, let alone the *E fornicatus* species complex, limits the proper identification and detection of parasitoids and predators that likely access the galleries during a very short timeframe.

While few parasitoid natural enemies of ambrosia beetles have been found, their implementation in biological control programs is fairly scarce (see Chapter 1). To date, the most effective approach towards forest pest management involves importation and inoculative release of promising natural enemies. However, the documentation to follow up on the establishment of biological control agents is poorly understood (Kulhavy and Miller, 1989).

An alternative, and less studied, natural control agent is nematodes. All nematode-insect associations can be categorized in one of three groups: phoretic, necromenic or parasitic. It is critical to determine the association between nematodes and the *E. fornicatus* species complex (Massey, 1974). Entomophathogenic nematodes garner the most interest in part due to their discernable effect and mortality on the host (Poinar, 1975). However, the most commonly reported nematode interaction with bark and ambrosia beetles is phoresy, in which the nematodes are nonlethal and their lifecycle does not strictly require the involvement of the beetle host (Giblin-Davis et al., 2013) The abundance of food, along with the favorable microhabitat of bark and ambrosia beetle galleries, facilitates the diverse establishment of phoretic nematode species. Yet, the true effect of phoretic, or seemingly-phoretic, nematodes is poorly understood in ambrosia beetles. At the very least, nematodes are biotic factors in beetle galleries that potentially regulate the beetle populations at an endemic level (Fettig and Hilszczański, 2015).

References

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.

Beaver, R. A., Wilding, N., Collins, N., Hammond, P., & Webber, J. (1989). Insect-fungus relationships in the bark and ambrosia beetles. *Insect-fungus interactions*, *121*, 143.

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

Borden, J. H. (1989). Semiochemicals and bark beetle populations: exploitation of natural phenomena by pest management strategists. *Ecography*, *12*(4), 501-510.

Browne, F. G. (1961). The biology of Malayan Scolytidae and Platypodidae. *Malayan Forest Records*, *22*(22), 1-255.

CABI. Invasive Species Compendiuum (2021). *Euwallacea fornicatus*. Available on https://www.cabi.org/isc/datasheet/18360453 accessed 15 March 2023.

Cook, D. C., & Broughton, S. (2023). Economic impact of polyphagous shot hole borer *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae) in Western Australia. *Agricultural and Forest Entomology*.

DeBach, P. (1964). Biological control of insect pests and weeds. *Biological control of insect pests and weeds.* 844 pp.

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 Disease*, *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 Disease*, *97*(7), 938-951.

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

Fettig, C. J., & Hilszczański, J. (2015). Management strategies for bark beetles in conifer forests. In *Bark beetles* (pp. 555-584). Academic Press.

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.

Giblin-Davis, R. M., Kanzaki, N., & Davies, K. A. (2013). Nematodes that Ride Insects: Unforeseen Consequences of Arriving Species. *Florida Entomologist*, *96*(3), 770-780.

Gomez, D. F., Skelton, J., Steininger, M. S., Stouthamer, R., Rugman-Jones, P., Sittichaya, W., Rabaglia, R. J. & Hulcr, J. (2018). Species delineation within the *Euwallacea fornicatus* complex revealed by morphometric and phylogenetic analyses. *Insect Systematics and Diversity*, *2*(6), 2.

Gomez, D. F., Lin, W., Gao, L., & Li, Y. (2019). New host plant records for the *Euwallacea fornicatus* (Eichhoff) species complex (Coleoptera: Curculionidae: Scolytinae) across its natural and introduced distribution. *Journal of Asia-Pacific Entomology*, *22*(1), 338-340.

Haack, R. A. 2003. Intercepted Scolytidae (Coleoptera) at U.S. ports of entry: 1985-2000. Integrated Pest Management Reviews, *1(6),* 253-282.

Hamilton, W. D. (1967). Extraordinary Sex Ratios: A sex-ratio theory for sex linkage and inbreeding has new implications in cytogenetics and entomology. *Science*, *156*(3774), 477-488.

Hulcr, J., Mogia, M., Isua, B., & Novotny, V. (2007). Host specificity of ambrosia and bark beetles (Col., Curculionidae: Scolytinae and Platypodinae) in a New Guinea rainforest. *Ecological Entomology*, *32*(6), 762–772.

Hulcr, J., & Stelinski, L. L.. (2017). The Ambrosia Symbiosis: From Evolutionary Ecology to Practical Management. *Annual Review of Entomology*, *62*(1), 285–303.

Jordal, B. H., Normark, B. B., & Farrell, B. D. (2000). Evolutionary radiation of an inbreeding haplodiploid beetle lineage (Curculionidae, Scolytinae). *Biological Journal of the Linnean Society*, *71*(3), 483–499.

Kirkendall, L. R., Biedermann, P. H. W., & Jordal, B. H. (2015). Evolution and diversity of bark and ambrosia beetles. In *Bark beetles*, 85-156. Academic Press.

Kulhavy, D., & Miller, M. C. (1989). Potential for biological control of *Dendroctonus* and *Ips* bark beetles. Nacogdoches, TX: Stephen F. Austin State University, Center for Applied Studies. 215 pp.

Massey, C. L. (1974). Biology and taxonomy of nematode parasites and associates of bark beetles in the United States. United States Department of Agriculture Handbook No. 446.

McPherson, G., Xiao, Q., Van Doorn, N. S., De Goede, L., Bjorkman, J., Hollander, A., Boynton, R. M., Quinn, J. F., & Thorne, J. H. (2017). The structure, function and value of urban forests in California communities. *Urban Forestry and Urban Gardening*, *28*, 43-53.

Mendel, Z., Protasov, A., Sharon, M., Zveibil, A., Yehuda, S. Ben, O'Donnell, K., & 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., & 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*, *102*(6), 1154–1164.

Paine, T. D., Raffa, K. F., & Harrington, T. C. (1997). Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annual review of entomology*, *42*(1), 179-206.

Rabaglia RJ, Dole SA, & Cognato AI. (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.

Smith, S. M., Gomez, D. F., Beaver, R. A., Hulcr, J. & Cognato, A. I. 2019. Reassessment of the species in the *Euwallacea fornicatus* complex after the rediscovery of the "lost" type specimen. *Insects*, *10*(9), 261.

Stouthamer, R., Rugman-Jones, P., Thu, P. Q., Eskalen, A., Thibault, T., Hulcr, J., & Zong, S. (2017). Tracing the origin of a cryptic invader: phylogeography of the *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae) species complex. *Agricultural and Forest Entomology*. *19*(4), 366-375.

Wickremasinghe, R. L., & Thirugnanasuntheram, K. (1980). Biochemical approach to the control of *Xyleborus fornicatus* (Coleoptera: Scolytidae). *Plant and Soil*, *55*, 9-15.

Chapter 1: Parasitoids associated with ambrosia beetles in the *Euwallacea fornicatus* species complex in Taiwan (Coleoptera: Curculionidae: Scolytinae)

Abstract

Ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) can occasionally be economically important due to the damage they can cause in agriculture, forestry and urban areas. While the majority of ambrosia beetle species attack dead or dying trees, a few species in the genera *Euwallacea*, *Xyleborus*, and *Xylosandrus* attack healthy host plants and are considered major pests. The concealed lifestyle of these beetles allows them to evade detection in exported wood material, which increases their chances of being introduced to non-native regions. Coupled with sib-mating, this concealed lifestyle also makes them difficult to control via chemical means like insecticides or pheromone trapping. Although a handful of studies have identified the natural enemies associated with ambrosia beetles, very few have done this with the goal of creating a classical biological control program. In this study, we sought to identify hymenopteran parasitoids associated with invasive ambrosia beetles belonging to the *Euwallacea fornicatus* species complex. We collected branches from infested trees in a native region in Taiwan, recorded parasitoid emergence, and attempted to rear any emerging parasitoids using laboratory reared beetles and experimentally infested logs. We identified three species of parasitoid wasp, one each in the families Bethylidae, Braconidae, and Eulophidae, that successfully parasitized beetles in the *E. fornicatus* species complex.

Introduction

Ambrosia beetles (Coleoptera: Curculionidae: Scolytinae and Platypodinae) can be economically important pests as a result of the extensive damage they can cause in agriculture, forestry and urban areas (Browne, 1961; Wood, 1982). Their cryptic lifestyle inside of traded wood and wood products, such as timber and nursery plants and wood packaging material, has resulted in many ambrosia beetles invading non-indigenous regions (Inward, 2020). The general behavior and habitat of ambrosia beetles varies little, and the vast majority of species live inside dead or moribund trees (Beaver, 1988; Hulcr et al., 2007). However, a few species, primarily in the genera *Euwallacea*, *Xyleborus*, and *Xylosandrus*, are considered pests and cause severe damage across a wide range of healthy or seemingly-healthy host plants (Kirkendall et al., 2015; Hulcr and Stelinksi, 2017). Once invasive populations have become established, few control measures are available as the beetles spend the majority of their time, even after reaching maturity, inside the xylem of the host plant. Their cryptic lifestyle makes the use of chemical control rarely feasible, with the exception of some semiochemicals that may control populations of a few species via push-pull management (Rivera et al., 2020; Byers et al., 2021).

Biological control is often more successful in stable agricultural systems such as orchards (DeBach, 1991) than in non-stable systems. Thus ambrosia beetles, as pests of stable forests environments might be good candidates for biological control. However, few studies have focused on their natural enemies (**Table 1.1**), and fewer yet have been done with the goal of establishing a biological control program. There are several factors

that likely contribute to the paucity of studies looking at the control of ambrosia beetles with parasitoids. Parasitoid wasps of ambrosia beetles have been commonly reported as non-existent or difficult to find, and consequently little has been done to assess their role in the control of their hosts (Dahlsten, 1982). They are difficult to detect largely due to the concealed nature of their beetle hosts compared to natural enemies that parasitize or predate on insects that live on readily accessible surfaces, such as foliage. In addition, it is difficult to observe natural enemies feeding on or parasitizing ambrosia beetles inside their galleries. The size of the host plant also may also present problems for collecting emerging parasitoids since it is not practical to contain an entire ambrosia beetle-infested tree. Despite these challenges, intensive studies were done to collect parasitoids of ambrosia beetles but were ultimately unsuccessful (Muraleedharan et al., 1988).

Few parasitoids of ambrosia beetles have been described **(Table 1.1)**. An explanation often given for this is the physical blocking of the gallery entrance, which subsequently halts further advancement of intruders, i.e. parasitoids and predators (Kirkendall et al., 2015). It is common in many species of ambrosia beetle to find an adult beetle sitting at the entrance using its abdomen to close off the entrance hole (Blackman, 1922; Kirkendall et al., 1997). Gallery entrances are also frequently blocked by a so-called “toothpick” or “noodle” consisting of compressed sawdust and frass being pushed out of the gallery. In some ambrosia beetle species, the larvae, usually late instars, partake in gallery protection by either moving frass to plug the entrance or by physically blocking the gallery using their whole body, also known as phragmosis (Strohmeyer, 1906, as cited in Kirkendall et al., 2015; Roberts, 1968).

Parasitoids have evolved several strategies to circumvent such blocking behavior and access their hosts inside the galleries. An obvious tactic would be to parasitize the "guard" beetle. An example of this behavior is found in some braconid species that parasitize adult ambrosia beetles. A detailed description of this behavior is found in Seitner and Notzel (1925), but in short, the female braconid uses her mandibles to grab an adult beetle at the entrance of its gallery, swiftly paralyzes it, and subsequently oviposits into the mesothorax of the beetle. The beetle regains mobility and resumes its normal activities, but eventually dies once the parasitoid larva reaches the last instar. Despite killing its host, the larvae have been reported to overwinter in the host beetle gallery, resulting in one to two generations per year. A similar strategy is adopted by eulophid wasps in the genus *Phymastichus*, which parasitize adult beetles as they initiate burrowing into the host plant or as they guard the gallery entrance (La Salle, 1990; Honsberger and Wright, 2022). Another tactic is to wait until the guard beetle deserts her post before entering the gallery. During early gallery development, the guard beetle cannot always remain at the entrance since gallery construction, fungal farming, and brood care depend solely on the colonizing beetle. *Cephalonomia stephanoderis* (Hymenoptera: Bethylidae), a parasitoid of the coffee berry borer *Hypothenemus hampei*, bypasses the blockade by entering the gallery while the foundress is busy with brood maintenance and further excavation. Once inside, the parasitoid feeds on eggs and early instars and may remain in the gallery until the preferred host stages (late instars or pupae) are present **(**Abraham and Moore, 1990).

Other parasitoids have solved the problem without having to gain access to the gallery. Some parasitoids belonging to the chalcid family Perilampidae do not oviposit directly in or on the beetle host. Instead, the females lay their eggs close to the gallery entrance, and upon hatching, the heavily sclerotized first instar larvae, called planidia, attempt to make their way into the gallery. This has been described for the parasitoid species *Monacon robertsi*, pupal ectoparasitoids of the platypodid ambrosia beetle *Crossotarsus barbatus* (Darling and Roberts, 1999). After entering the gallery, the planidia initially feed on eggs and young larvae, and later enter pupating beetles to complete their development. However, success is not guaranteed, and many of the planidia fail to find or reach a host and are expelled from the gallery opening along with sawdust and frass. Finally, some parasitoids have been reported to parasitize their hosts by using their ovipositor to drill through the bark to reach the beetles inside the gallery (Kalshoven, 1960). This was found in a parasitoid belonging to the eulophid genus *Aprostocetus* (Roepke, 1914, as cited in Kalshoven, 1960), and is restricted to parasitizing *Xylosandrus morigerus* beetles found in twigs. This enables them to reach the hosts inside the galleries, but only when the beetles are present in thin branches.

While natural enemies in the form of parasitoids have been found, they have rarely been applied for the control of ambrosia beetles. One simple reason for this is that many non-native ambrosia beetles are not considered an economic or ecological concern because they attack dead or dying trees. However, there are a small number of ambrosia beetle species that are economic pests, and as such have received greater attention. One such invasive species is the redbay ambrosia beetle (RAB), *Xyleborus glabratus*

(Curculionidae: Scolytinae), which is the primary vector of the pathogenic fungus *Raffaelea lauricola*, the causal agent of laurel wilt disease in Lauraceae plants across the southeast region of the United States (Brar et al., 2012). Surveys conducted with naturally infested and sentinel logs, in both native and non-native regions, suggest the presence of a few possible parasitoid wasps of RAB, e.g. *Tetrastichus* sp. (Eulophidae). However, the majority of parasitoids encountered could not be identified past a family level and could be parasitoids of other insects associated with the trap logs (Peña et al., 2015). While some of these parasitoids may display host specificity toward RAB, no studies have been done to prove this or determine their potential towards biologically controlling this beetle.

The present study focuses on parasitoids of a group of closely related, and morphologically very similar, invasive ambrosia beetles that have recently established damaging populations in parts of the US: the *Euwallacea fornicatus* species complex (Stouthamer et al., 2017). Two species, the polyphagous shothole borer (PSHB) (*E. fornicatus*) and the Kuroshio shothole borer (KSHB) (*E. kuroshio*), have invaded California and have been targeted for biological control. These species attack healthy trees and vector fungi in the genus *Fusarium* that are the causal agents of *Fusarium* dieback (Eskalen et al., 2013). Shothole borers (SHB) were initially thought to be a threat only to urban forests (street trees) and agriculture (particularly avocado) (Eskalen et al., 2013). However, in 2015 it became clear that riparian areas could also be very susceptible to attacks from these pests with the destruction of an estimated 287,620 willow trees, both arroyo (*Salix lasiolepis* Benth.) and Goodding's black (*Salix gooddingii* C.R. Ball),

in the Tijuana River Valley in San Diego County (Boland, 2016). In addition to California, PSHB has also invaded Hawaii, Israel, South Africa, and, more recently, Australia (Rugman Jones et al., 2021; Mendel et al., 2012; Paap et al., 2018; https://www.outbreak.gov.au/current-responses-to-outbreaks/polyphagous-shot-hole-borer; accessed December 27, 2022) A third member of the complex, the tea shothole borer (TSHB) (*E. perbrevis*) was also established in Hawaii as early as 1910, but has also recently established in Florida (Swezey, 1941; Rabaglia et al., 2006; Rugman Jones et al, 2021). While chemical control has been explored through active use of various insecticides, fungicides, and rhizobacteria to manage beetle infestations, the methods are costly and require further investigation on multiple host plants and larger field applications (Eatough Jones et al., 2017; Eatough Jones and Paine, 2018; Guevara-Avendaño et al., 2018). Biological control is one of the few viable options in part due to the widespread occurrence of these pests, in both urban and sensitive riparian areas. Herein, we identify the community of parasitoids associated with the *E. fornicatus* species complex in part of its native range (Taiwan) where all three of the species PSHB, KSHB, and TSHB co-occur (Stouthamer et al., 2017).

Materials and Methods

Collection of SHB-infested logs in Taiwan

Material for this study was collected at 5 different times, from January 2017-October 2019, across multiple SHB-inhabited avocado orchards, in the Danei district of Tainan, Taiwan. Branches of approximately 7-11 cm diameter, and with physical signs of

boring activity (frass) and multiple infestation points (at least 10 gallery holes), were removed from avocado trees, and nearby castor bean trees, and cut into 20-25 cm long logs. Any heavily infested twigs (< 5 cm diameter) and larger diameter bolts were also collected. The cut ends of the logs were sealed with paraffin wax to reduce desiccation and maintain suitable growth conditions for any concealed insects. Logs were subsequently shipped to the University of California, Riverside (UCR) Insectary and Quarantine (I&Q) facility under USDA-APHIS permit (P526P-19-02285).

Collection of parasitoids from imported wood

Within I&Q, imported logs were individually caged in either lidded clear plastic containers [36 cm (H) x 11 cm (D) (Container and Packaging Supply, Inc., Eagle, Idaho, USA)] or in lidded mason jars (1 qt). The center of the mason jar lids was removed and replaced with stainless steel bolt cloth (SKU # 070X070T0037; TWP, Inc., Berkeley, CA, USA), to prevent SHB and other insects from escaping while providing ventilation to minimize condensation and the growth of fungal and other microbial contaminants. Similarly, a 10 cm diameter disc was cut out of the lids of the plastic containers and replaced with steel bolt cloth. The containers were housed in a rearing room (28^{o}C +/- 2, 50% RH) and checked for parasitoid wasp emergence every morning, afternoon, and evening until emergence ceased. Each emerged parasitoid was collected with either a soft brush or an aspirator (depending on size) and introduced to experimentally-infested SHB logs in an attempt to initiate colonies of the parasitoids (see Section 2.4). Any emerged parasitoids that died during shipment, between daily monitoring times, or after a rearing attempt, were preserved in 95% ethanol for identification.

Identification of parasitoid wasps

Parasitoids were initially sorted into morphotypes under a dissecting microscope. The DNA of a representative set of individuals of each morphotype was extracted using a non-destructive HotSHOT method (Truett et al., 2000). Specimen carcasses were subsequently retrieved for imaging and morphological assessment/confirmation. A fragment of the mitochondrial gene cytochrome oxidase c subunit 1 (COI) was amplified via PCR with the universal forward primer LCO1490 and reverse primer HCO2198 following Stouthamer et al. (2017). The D2 region of the nuclear ribosomal 28S (28S-rDNA) also was amplified using the forward primer 28sF3633 and reverse primer 28sR4076 (Stouthamer et al., 2017). Amplified PCR products were cleaned with the Zymo DNA Clean & Concentration- 5 kit (Zymo Research Corporation, Irvine, CA, USA) and sequenced in both directions at the Institute of Integrative Genome Biology, UCR. Forward and reverse reads were checked for parity, trimmed, and aligned, using the software BioEdit 7.0.5.3 (Hall, 1999). COI sequences were collapsed into haplotypes with DnaSP (Librado and Rozas, 2009) and each haplotype was subsequently subjected to a BLASTn search of the NCBI sequence database (Altschul et al., 1990) and queried against BOLD using the BOLD identification engine (Ratnasingham and Hebert, 2007). In the case of COI, BLASTn and BOLD inquiries returning a match of 97% or above with a named species, were assigned to that species. COI sequences returning a match below 97% were identified to an appropriate taxonomic level (family, subfamily, or genus) inferred from the results of the search. Species identity was also inferred from

28S-D2, but being a more conserved locus, it was largely used to support or refine the COI designation, except on the occasion that a BLASTn search returned a 100% match.

The taxonomic position of the different parasitoids was estimated using maximum likelihood centered on COI sequences. Twenty-one representative individuals were used for the phylogenetic analysis with an additional 33 accessions from within each identified group including one outgroup obtained from GenBank® and BOLD. The General Time Reversible model (GTR) with 5 discrete gamma categories (+G) was determined as the best fit model for maximum likelihood using the Model Test in MEGA X (Kumar et al., 2018; Stecher et al., 2020). A phylogenetic tree was generated using the customized, or "Advanced," option in the integrated phylogenetic analysis workflows provided by NGPhylogeny.fr where "DNA" or "Nucleic Acid" were manually selected in the "data type" and "sequence coding" criteria (Lemoine et al., 2019). The generated phylogenetic tree was later edited on FigTree v1.4.1(Rambaut, 2012).

Specimens of all parasitoids for which a colony was initially established, or which showed clear signs of association with SHB beetles (e.g., entering SHB galleries) were subject to a more detailed morphological examination. After being dried overnight using hexamethyldisilazane (HMDS) (Heraty and Hawks, 1998) individual specimens were held in place in a small watch glass with a drop of lubricant gel. Layered images of key morphological characters were captured using a Leica M10 with a Z16 APO A microscope and stacked using Zerene Stacker (ver. 104, Zerene Systems, LLC). A genus level identification then was sought from expert taxonomists (see Results).

Introduction of newly emerged parasitoids to lab-reared PSHB wood material

In preparation for receiving imported parasitoids from Taiwan, we attempted to replicate a "natural" rearing setup similar to what might be expected in the native environment of the parasitoids, in an effort to: 1) optimize the chances of successful parasitism, and 2) confirm an explicit parasitoid-host relationship by proving that offspring can develop from logs infested only with PSHB. This was done by infesting freshly cut 25-30 cm long avocado and castor bean logs with 200-400 PSHB from lab colonies, originally initiated using beetles that emerged from infested castor bean trees in Riverside County (Riverside, CA), and maintained on semi-artificial sawdust media (Carrillo and Dodge et al., 2020). Infestation of newly collected logs was done weekly beginning four weeks prior to arrival of shipments from Taiwan and ceased once no more parasitoids emerged in I&Q. The different time points of infestation allowed the availability of all beetle life stages upon introduction of potential SHB parasitoids.

During each daily check on the imported logs, any emerged parasitoid wasps were collected in 33 ml plastic vials (9 Dram) [BioQuip Products, Inc., Rancho Dominguez, CA, USA]. To account for the variable feeding preferences of emerging parasitoids, we provided a variety of food sources: honey, sugar water, rehydrated raisins, and pollen. Cotton swabs briefly dipped in water were also placed at the bottom of each vial, to recreate the high humidity found in Tainan, Taiwan. Following an opportunity to feed (~30 mins), parasitoids were sexed, and whenever possible, male and female pairs were introduced to an insect cage containing a PSHB-infested castor bean log and provided with further food for sustenance (see above). After an hour, pairs were relocated to a different cage with a PSHB-infested avocado log and food. This process was repeated on

logs with different PSHB-infestation points to maximize the exposure of parasitoids to available hosts. Each experimental log was subsequently maintained in a separate container and observed daily for parasitoid offspring emergence. Excess females were also introduced to infested logs with the assumption that they would at least produce haploid male offspring. Excess males were maintained in the growth chamber in the hope that a female mate would be collected before they died. Any emerging offspring were again collected and introduced to new PSHB-infested logs in an effort to establish an ongoing lab colony.

Once the F1 generation of parasitoids stopped emerging from the experimental logs (> 9 weeks), they were further examined for signs of parasitism. Gallery entrances were checked for the vacated carcasses of SHB hosts (**Figure 1.1**), and two logs from each parasitoid species were carefully split and deconstructed in search of remnant wasp cocoons and/or beetle carcasses. To dissect the log, it was placed in a bench vice and split into smaller sections using chisels.

Attempts were also made to rear the three most abundant (and relevant; see Results) parasitoids, using PSHB colonies contained within the semi-artificial media. Three individuals of each parasitoid species were each placed in a 50 mL PSHB lab colony tube and switched every 15 minutes to a progressively older PSHB colony tube, facilitating exposure of the parasitoid to PSHB colonies aged 0 days (introduced at the same time as the parasitoid), 7 days, 14 days, 28 days, 36 days, and 48 days old. During this period, any notable behavior of mating, host finding and/or parasitism was documented and recorded on camera when possible.

Results and Discussion

Overview

In this study, we encountered many parasitoids associated with host plant material infested with beetles of the *E. fornicatus* cryptic species complex. DNA sequences obtained from the parasitoid wasps emerging from the logs collected in Taiwan were identified via BLASTn searches as belonging to the following hymenopteran families: Aphelinidae, Bethylidae, Braconidae, Crabronidae, Encyrtidae, Eulophidae, Platygastridae, Pteromalidae, and Torymidae **(Table 1.2).** Twenty-one haplotypes were detected among the COI sequences, but most were identifiable only to family level (**Table 1.2; Figure 1.2**), and only three species (one each in the families Bethylidae, Braconidae, and Eulophidae) were proven to parasitize individuals of the *E. fornicatus* cryptic species complex (**Table 1.3**). Each is discussed in further detail below, but will be formally described elsewhere.

Eucosmophorus sp. (Braconidae: Euphorinae):

The first parasitoid wasp to have been successfully reared on PSHB-infested logs was a new braconid species in the genus *Eucosmophorus* (formerly *Sinuatophorus* prior to being synonymized (Stigenberg et al., 2015)). Both COI and 28SD2 sequences initially suggested the parasitoid may belong to the genus *Cosmophorus* (Hymenoptera: Braconidae). However, morphological assessment by J. Stigenberg (Swedish Museum of Natural History), an expert taxonomist of Euphorinae, confirmed its identity as *Eucosmophorus*.

Morphology. Dimorphism in color and morphology clearly distinguish the males and females. The length, measured from the center of the head to the furthest tip of the abdomen, of females ranged from 3.51-3.79 mm (n = 7) with a mean of 3.64 ± 0.104 mm. Males were slightly smaller than females and ranged between 3.36-3.58 (n = 5) with a mean of 3.44 ± 0.08 mm. Females are mostly dark brown/black with hues of light brown at the tip of the legs and mandibles **(Figure 1.3a**), while the head and thorax of males are ubiquitously light brown with dark brown/black abdomens **(Figure 1.4)**. Noticeable are the enlarged, overlapping and multidentate mandibles found on the females **(Figure 1.3a)** in addition to the thick sheath covering a characteristic ovipositor (**Figure 1.3b)**, which for this genus, is described as "wavy" (Quicke, 2015). This wavy ovipositor is thought to act as a steering mechanism utilized by the parasitoid inside the host gallery, though this function has not been explicitly confirmed.

Developmental biology and mating behavior. The developmental time, calculated from the time of initial exposure of the parental wasps to PSHB-infested logs until the emergence of adult offspring, for *Eucosmophorus* sp. ranged from 37-43 days with males recorded to emerge 24 to 72 hours prior to females. One recorded exception was an experimental log from which 8 males emerged 12 days before the first female. In rare instances, females emerged before the males, or without a male, and were left unmated. Copulation of *Eucosmophorus* parasitoids was observed on a few occasions but was somewhat unpredictable. After females were paired with a male in our rearing attempts, mating occurred either immediately or not until several hours later. Similar to most braconids (Avila et al., 2017), courtship behavior was initiated with brief sessions of

wing-fanning from the male, presumed to either waft the female pheromones towards it (Vinson, 1972b), or to generate vibrational signals to nearby females (Villagra et al., 2011). The motion gradually increased within close proximity of the female. Between bouts of wing-fanning, males groomed their hind legs together followed by their abdomen, which occasionally moved in an upwards motion. During this male "display", females either remained stationary or also engaged in grooming their hind legs, antennae, and ovipositor. After this initial grooming phase, the male approached the female by tapping his antennae on her abdomen, and attempted to mount her with both wasps facing the same direction. The female remained in place if she was receptive to the male's mating approach or refused him by walking away. A "jilted" male either persisted with the same female until mating was achieved, or simply moved on or pursued another female. In successful mating, the male continued to antennate the female's abdomen until the aedeagus was inserted in the female's genital orifice. Copulation was terminated by the male, which immediately started to groom himself, while the female, after a brief moment of stillness, proceeded to groom her ovipositor. The duration of copulation ranged from 43.28 to 72.09 seconds (n = 4).

Host searching behavior. Females placed into cages along with PSHB-infested logs were observed antennating the surface of those logs, focusing most on areas in front of gallery entrances. Females that entered a gallery always did so face forward, and re-surfaced in the same orientation (i.e. abdomen first). Of the six individual females carefully monitored, they spent spend between 11.61- 32.49 seconds inside the galleries. Upon emergence, females immediately began to groom their antennae and ovipositor, the

latter consistently observed to move in either an up-down or side-to-side motion upon reemergence. Grooming and observed upwards movement of the abdomen occurred periodically during host searching. While only female wasps were observed entering beetle galleries, we were able to document the initial emergence of a male *Eucosmophorus* from an avocado branch. Similar to their behavior on the experimental logs, mated females placed in semi-artificial media tubes with active beetle colonies showed some interest and were observed entering the galleries if left in the tube longer than the initial proposed time of 15 minutes. A female parasitoid that remained in an inverted tube for approximately 30 minutes was noted to enter the gallery twice. Fortunately, the gallery was excavated against the side of the tube, which allowed us to observe the wasp's behavior inside the gallery. Upon its entrance, the female wasp encountered a beetle and struggled to grasp it with her mandibles as the beetle persisted to push her away. Despite the observation of excitatory behavior, i.e. antennating the host and excessive grooming of ovipositor, inside the gallery, neither parasitism nor parasitoid offspring emergence was observed from beetle artificial tubes.

Potential value as a biological control agent. While parasitism of bark beetles is common in Braconidae (Marsh, 1979), parasitism of concealed hosts such as ambrosia beetles seems largely limited to the subfamily Euphorinae (Stigenberg et al., 2015). This appears to be the case in this study as we were able to rear at least one offspring generation on PSHB lab-infested logs from 3 out of the 5 shipments received, with one reaching a second offspring generation **(Table 1.3)**. From the material originally imported from Taiwan, *Eucosmophorus* wasps almost exclusively emerged from infested

avocado wood, the only exception being a single male from a castor bean log. In contrast, the majority of successfully reared offspring emerged from PSHB-infested castor bean logs. Within the logs from which the first offspring generation of *Eucosmophorus* emerged, destructive sampling (conducted after emergences ceased) revealed the presence of multiple thin, translucent cocoons in the SHB galleries, one of which was found next to a dead adult PSHB beetle **(Figure 1.5)**. A dead female wasp, presumably from an unsuccessful eclosion, was found near the cocoon (**Figure 1.5c**). Following emergence from the imported logs, males and females were very short-lived despite being provided access to several different sugar and protein-rich food sources. Their already short lifespan declined further when reared on our experimentally infested logs. Female adults from the first generation generally lived for 2-3 days, while female adults from subsequent generations lived for only 1-1.5 days. Similarly, F1 and F2 adult males lived for around 5 and 2 days, respectively.

In comparison with the other parasitoids collected, *Eucosmophorus* was the only parasitoid species that was consistently encountered across all shipments. Yet despite 301 recorded emergences from imported wood, failure to rear a continuous colony in large numbers clearly demonstrates that the biology and parasitism behavior of this species is poorly understood. What we expected to find was inferred mainly from a single publication that describes the life history of a close sister taxon *Cosmophorus cembrae*, an endoparasitoid of the bark beetle *Pityophthorus henscheli* (Coleoptera: Scolytinae), in which females grab their hosts by the head reaching under the beetle to paralyze and oviposit (Seitner & Notzl, 1925). Direct parasitism by *Eucosmophorus* was not observed

in our study, though a female wasp was observed attempting to attack a female PSHB near the surface of a gallery entrance, but without the use of her mandibles. When the PSHB retreated into her gallery, the wasp pursued the beetle into the gallery and quickly resurfaced. This would appear to differ from the head-to-head position adopted by *C. cembrae* as described by Seitner and Notzl (1925). Both species entered the host gallery facing the entrance, yet *Cosmophorus* wasps were described to exit anteriorly with their head first while *Eucosmophorus* wasps resurfaced posteriorly suggesting that the latter may parasitize the beetles from behind. A possible explanation for the low number of emerging offspring could be the failure of parasitoid larvae to pupate in a favorable orientation relative to the gallery exit. Shaw and Berry (2005) discuss the importance of anterior versus posterior, caudal, exits of *Cryptoxilos thorpei* (Euphorinae) larvae from their wood-boring scolytine hosts. Anterior, or tunnel forward, exits confined the larvae in the gallery, and prevented them from emerging successfully. The failed emergence was a result of the beetle carcass, and potentially the parasitoid's own cocoon, which blocked their path. On the other hand, parasitoids that exited posteriorly allowed the larvae to pupate closer to the gallery entrance with little to no difficulties encountered upon emergence. We were able to document an empty *Eucosmophorus* cocoon formed posteriorly from a PSHB carcass suggesting that a similar exit strategy is favored with this species.

Another factor that added difficulty to establishing a colony of *Eucosmophorus* was the high (and increasing) male to female sex ratio in successive offspring generations. At least two factors may have contributed to this. First, our rearing setup

may have failed to provide optimal conditions conducive to mating between the sexes, resulting in unmated females producing haploid sons. Secondly, *Eucosmophorus* may exhibit complimentary sex determination (CSD), a phenomenon found in multiple species of Braconidae. In haplodiploid organisms, under CSD, fertilization of the egg is not enough to ensure the production of a female offspring: the diploid offspring must also be heterozygous at some sex determining locus (or loci). This is problematic since inbreeding is much more likely to occur in small laboratory colonies (i.e. our rearing attempts), not including the initial bottleneck from the small number of parasitoids emerging from the limited materials collected, which in turn induced the likelihood of homozygous offspring, and the production of diploid males from fertilized eggs (Stouthamer et al., 1992; Cook and Crozier, 1995; Cowan and Stahlhut, 2004). These diploid males are typically sterile, or at least have reduced fitness (Petters and Mettus, 1980). Since solitary female braconids generally mate once (Quicke, 2015), mating with a diploid male that is infertile would cause the subsequent generation to all be males. Also, among the COI sequences obtained from 25 individual *Eucosmophorus* adults (both parental and lab-reared offspring), only one haplotype was identified. This suggests that the species is either not native to Taiwan, or any variation in COI may have been wiped out by cytoplasmic incompatibility (CI)-inducing *Wolbachia* (Stouthamer et al., 1999). If *Wolbachia* infection is not ubiquitous across all *Eucosmophorus* wasps, a *Wolbachia*-free female mating with an infected male would result in an increased male production of the subsequent population. While cytoplasmic incompatibility is generally uncommon in ichneumonids (Quicke, 2015), it may warrant further investigation.

In continuing research efforts, we recommend increasing the arena size to provide the wasps multiple logs with large numbers of adult beetles to parasitize. Although CSD was not confirmed in this study, we strongly recommend grouping females with males emerging from different regions to minimize the risk of diploid males in subsequent colonies. This also would be beneficial in terms of reducing the genetic bottleneck typically associated with lab colonies. Overall, further exploration of parasitism behavior and the developmental biology and genetics of *Eucosmophorus* wasps is needed to determine their full potential as biological control agents against the *Euwallacea fornicatus* species complex.

Plastanoxus sp. (Bethylidae: Scleroderminae)

The second parasitoid wasp to have successfully produced an offspring generation in our studies was a new species in the family Bethylidae. Sequences of the COI closely matched those of an unidentified bethylid species caught in a malaise trap in Pakistan [KY835482], and sequences of 28S-D2 suggested it may be close to *Cephalonomia formiciformis* (Bethylidae: Scleroderminae) [KC762949]. Subsequently, morphological assessment in conjunction with an authority on bethylid taxonomy, C. Azevedo (Ph.D., Federal University of Espirito Santo, Brazil), placed the wasp in the genus *Plastanoxus*.

Morphology. There were no apparent signs of sexual dimorphism to distinguish the males and females, contrary to what has been previously reported for other members of the family (Azevedo et al., 2010). Size ranged from 2.1 - 2.3 mm (n = 3) with the body being black/dark brown with hues of light brown on the legs, antennae, and mandibles **(Figure 1.6).**

Developmental biology and mating behavior. Parasitoids introduced to PSHB infested logs were either observed entering beetle galleries, or not found inside the container the next day, suggesting they might have entered the galleries sometime overnight. In one occurrence, two adult parasitoids resurfaced briefly only to re-enter the same gallery. Mating behavior was not observed and is presumed to take place within the gallery. Longevity of the parasitoids could not be determined as wasps did not resurface in the days that followed, with only a handful being found dead at the bottom of rearing containers several weeks later.

Host searching behavior. No obvious host searching behavior was observed in wasps confined with the experimental rearing logs, and it is believed that after entering a gallery, the wasps remain there for some time. Wasps placed in tubes with beetles reared on semi-artificial media displayed initial signs of interest as one partially entered the gallery but eventually retracted and remained on the surface. That same wasp was then placed in a different tube with a deconstructed beetle gallery containing multiple PSHB late instars and pupae. Although no direct parasitism was observed, we did observe excitatory behavior of excessive grooming and curvature of the abdomen directed at a beetle larva.

Potential value as a biological control agent. While neither mating nor parasitism was observed, emergence of an F1 generation, though low in number, was recorded from a PSHB-infested avocado log introduced only to *Plastanoxus* parasitoids (**Table 1.3**). With over 2,200 species worldwide, bethylids (flat wasps) are known to be ectoparasitoids of both the larvae and pupae of a range of Coleopteran and Lepidopteran

hosts (Gordh and Moczar, 1990). With particular relevance to bark and ambrosia beetles, females of the bethylids *Cephalonomia stephanoderis and Prorops nasuta* paralyze and oviposit on fully developed larvae, prepupae, pupae, and occasionally pharate adults (Portilla, 1999) of the coffee berry borer (CBB) (Abraham and Moore, 1990). Furthermore, many species of adult bethylids are known to directly feed on their hosts (Lauziere et al., 2001), and, after oviposition, remain in the concealed environment of their host where food, in the form of younger larval instars (which are not favored for offspring development), is plentiful (Clausen, 1940). Currently there are 14 described species in the genus *Plastanoxus*, with one, *Plastanoxus westwoodi*, intensively studied as a biological control agent of the stored product pest the flat grain beetle, *Cryptolestes pusillus* (Rahman and Islam, 2006).

Despite other species of *Plastanoxus* being reported as being both aggressive predators (on younger stages of host) and strategic parasitoids (of suitable older stages of host), the low offspring emergence recorded in this study again illustrates a shortfall in our knowledge of the biology and ecological habits/requirements of this new *Plastanoxus* sp. One possible factor behind the lack of rearing success could be a result of agonistic, or aggressive, behavior observed in some bethylid species over access to food. Cannibalism has been reported in bethylids, with females occasionally eating their own eggs when food sources become scarce, thereby reducing brood size, (Clausen, 1940). In our set up, deterioration of the field-cut and experimentally infested logs, post-collection or post-initiation, respectively, may have resulted in a nutritionally poor environment for both the female wasp and her offspring, thereby affecting emergence and survival. The

paraffin wax sealed logs provided to the wasps were not intended to maintain their water content indefinitely. It is easy to imagine a scenario in which desiccation (of a log) leads to reduced growth of the ambrosial fungi (the sole food source of the SHB), which eventually results in SHB brood reduction or elimination, and in turn, death of the adult parasitoid due to absence of hosts on which to feed. Further sampling and inquiry are much needed to make any conclusive statements on the biology of these wasps.

For future studies on rearing bethylid wasps, we recommend using a larger diameter beetle host plant to maximize water retention and adding the wasps when second instar beetles are abundant to be fed on by female parasitoids. Work has been done that suggests that it may be possible to rear *Plastanoxus* species on alternative hosts such as the rice weevil *Sitophilus oryzae* (Coleoptera: Curculionidae) (Howard et al., 2002). This might be worth pursuing.

Phymastichus sp. (Eulophidae: Tetrastichinae)

The third parasitoid wasp successfully reared on PSHB-infested logs is a new species in the family Eulophidae. Initially, BLASTn inquiries of both 28S and COI sequences closely matched a wasp in the genus *Aprostocetus* (Eulophidae: Tetrastichinae). A very recent deposition of sequences belonging to a newly described species in the genus *Phymastichus* from Hawaii (Honsberger and Wright, 2022) provides further support to its identification.

Morphology. Based on molecular identification, male and female specimens were given to R. Burks (Ph.D., University of California, Riverside), expert taxonomist of Eulophidae, for morphological examination. This morphological assessment determined

that the specimens belonged to an as yet undescribed species in the genus *Phymastichus*. The antennae of male parasitoids lack a sensory plaque located on the ventral side of the scape. The presence of this plaque is a unique and distinguishable key characteristic only found in the subfamily Tetrastichinae, with the exception of the genus *Phymastichus*. Sex ratio of nearly 1 male to 1 female was observed in parental and subsequent filial generations. Strong sexual dimorphism was apparent in size as females (**Figure 1.7**) ranged between 1.58 - 1.66 mm (n = 2) and the males **(Figure 1.8)** were significantly smaller with a range of 0.86 – 0.93 mm (n = 2).

Developmental biology and mating behavior. The developmental time for the *Phymastichus* parasitoids ranged from 28-32 days where both females and males were sighted in the container together on the day of emergence. The adult longevity of this species is between 4 to 5 days (n = 10). Offspring generations emerged from PSHB-infested castor bean logs, while parental emergence was mainly observed from shipped avocado logs. Parasitoid emergence was not reported from any imported castor bean logs. Mating was not observed between males and females though it has been suggested that mating may take place inside the host carcass prior to emergence (Espinoza et al., 2009). That said, Honsberger and Wright (2022) report differently for *Phymastichus holoholo* with sighting of male courtship behavior and mating.

Host searching behavior. Female *Phymastichus* wasps were seen antennating the wood surface near beetle gallery entrances. While direct parasitism was not observed, adult PSHB carcasses were found at the gallery entrance with exit holes located on the elytral declivity **(Figure 1.1)**, which is characteristic of this genus. Furthermore, the

galleries appeared to be in the early stages of excavation suggesting that the host died not too long after being parasitized. Rearing modifications were made by combining *Phymastichus* parasitoids in cages with early PSHB-infested logs along with PSHB beetles that had not entered the logs. Attempts at rearing *Phymastichus* wasps on beetles reared in semi-artificial tubes were unsuccessful.

Potential value as a biological control agent. Records of emergence from three subsequent offspring generations makes this parasitoid the longest lab reared natural enemy of the *E. fornicatus* species complex in our study. Of the 153 avocado logs received on May 2017, a total of 24 (f = 13, m = 11) *Phymastichus* wasps emerged from 7 logs **(Table 1.3)**. Currently, there are three described species in the genus *Phymastichus*: *P. coffea* and *P. xylebori*, (La Salle 1990 and 1995, respectively), and *P. holoholo* (Honsberger and Wright, 2022), all of which shared the common feature of leaving behind an exit/emergence hole on the elytral declivity of their beetle host. *Phymastichus* is the only genus in Tetrastichinae (~3000 spp.) to attack the adult stage of their host (LaSalle, 1995; Honsberger and Wright, 2022). Of the three described species of *Phymastichus*, only *P. coffea* has been extensively studied as a biological control agent, against CBB. Female wasps will parasitize adult CBB and oviposit two eggs, one male and one female. Espinoza et al. (2009) stated that the highest rate of parasitism occurred as CBB beetles initiated burrowing into the coffee berry, though parasitism can still be seen 7 days after CBBs have burrowed. More recently, the newly described *P. holoholo* was reported to parasitize *Xyleborus ferrugineus* and *Xyleborus affinis* in large numbers. Honsberger and Wright (2022) reported two instances of *P. holoholo* parasitism

on PSHB and TSHB beetles, though they suggest it being a possible case of accidental parasitism considering the fully developed adult wasp was found inside the dead SHB beetle without an emergence hole.

In this study, *Phymastichus* wasps collected in May 2017 were successfully reared for the longest time period of all 3 parasitoids evaluated, yet total offspring emergence declined in number with subsequent generations despite applying proper modifications to optimize rearing. Further studies on the parasitoid biology and host preference are necessary for further assessment in a biological control program. For future studies involved with rearing this parasitoid, we recommend using smaller semi-artificial arenas composed of beetle diet topped with cut out disks of logs (preferably from a host plant with high water retention). In the past, we have documented PSHB beetles to burrow through thinly sliced avocado disks and proceed digging into the diet. This presents a favorable setup for the *Phymastichus* wasp to parasitize the host in the early stages of gallery excavation. If successfully mass-reared on SHB, their potential as biological control agents is promising given their preference for parasitizing beetles in the early stage of gallery excavation, which not only kills the adult prior to oviposition, but also prevents both physical and fungal damage in the host plant.

Other parasitoid species

Among the other parasitoids collected during this multi-year study, the specific identity and host preferences of the majority remain unknown. However, their documentation and identification to at least family, is not without merit. Initially, emergence of Encyrtidae and Pteromalidae parasitoids was regarded positively as reports

of parasitism, mainly from Pteromalidae, have previously been documented on some scolytine beetles (Kenis et al., 2004; Kirkendall et al., 2015). However, parasitism on PSHB was not observed in this study. Indeed, the two encyrtid wasps encountered, *Thomsonsica* sp. and *Comperiella bifasciata*, are both known to be parasitoids of armored scale insects (Diaspididae) (Qin et al., 2017). Although no scales were collected from the Taiwanese logs, it seems almost certain that one or more were present. *Polemistus* (Crabronidae) emergence was recorded across most shipments, but in small numbers. An adult *Polemistus* was also observed in an old, uninhabited gallery of a split log with no apparent signs of shothole borers being present. This appears to be in line with previous reports that *Polemistus* species do not interact with scolytine beetles as parasitoids, but instead utilize dead wood and old galleries created by the beetles for their own nesting purposes (Bohart and Menke, 1976). Numerous bethylid species emerged from the native SHB-infested wood. Based on phylogenetic analyses **(Figure 1.2)**, we can infer that in addition to *Plastanoxus* sp. six other bethylid species emerged from the native SHB-infested wood with the majority collected from a single log. Furthermore, none of the sequences obtained from these species matched an unknown bethylid wasp that was extracted from a parasitized shothole borer during a separate collecting trip in Taiwan 2015 **(Figure 1.9)**. This suggests that bethylid species may commonly be associated with the *E. fornicatus* cryptic species complex. However, correlation is not necessarily causation, and the native logs infested by the target beetles also harbored other insects, i.e. moths and flies. Like the two encyrtids, it is likely that some of the remaining parasitoids were not associated with the shothole borers, but with other insects that

emerged from the logs, making it difficult to explicitly link an emerging parasitoid to an ambrosia beetle host. None of these other bethylids were successfully reared on our experimental logs. Furthermore, these parasitoids were encountered in such low numbers to suggest that even if they do occasionally parasitize SHB, they are likely not a significant source of mortality.

Conclusion and future directions

Mass-rearing parasitoids of concealed hosts faces many challenges, and is frequently dismissed in large part due to the concealed association. Our study is the first to find, identify, and better understand the trophic interactions and biology of natural enemies explicitly associated with the *E. fornicatus* species complex. Further information about the relationship of these (and other) parasitoids with their concealed hosts might be obtainable through gut content analysis. We also recommend expanding the sampling region in Taiwan to increase the collection range of natural enemies in the region.

References

Abraham, Y., Moore, D., & Godwin, G. (1990). Rearing and aspects of biology of *Cephalonomia stephanoderis* and *Prorops nasuta* (Hymenoptera: Bethylidae) parasitoids of the coffee berry borer, *Hypothenemus hampei* (Coleoptera: Scolytidae). *Bulletin of Entomological Research*, *80*(2).

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

Avila, G. A., Withers, T. M., & Holwell, G. I. (2017). Courtship and mating behaviour in the parasitoid Wasp *Cotesia urabae* (Hymenoptera: Braconidae): mate location and the influence of competition and body size on male mating success. Bulletin of Entomological Research 107, 439–447.

Azevedo, C. O., Alencar, I. D. C. C., & Barbosa, D. N. (2010). Order Hymenoptera, family Bethylidae. *Arthropod Fauna of UAE*, *3*, 388-411.

Beaver, R.A. (1988), Biological studies on ambrosia beetles of the Seychelles (Col., Scolytidae and Platypodidae). Journal of Applied Entomology, 105: 62-73.

Blackman, M. W. (1922). *Mississippi bark beetles* (Vol. 11). Mississippi agricultural experiment station.

Bohart, R. M., & Menke, A. S. (1976). *Sphecid wasps of the world: a generic revisio*n. University of California Press, Berkeley, California.

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

Brader, L. (1964). Etude de la relation entre le scolyte des rameaux du caféier, *Xyleborus compactus* Eichh. (*X. morstatti* Hag.), et sa plante-hote. Mededelingen Landbouwhogeschool, Wageningen, 64:1-109.

Brar, G. S., Capinera, J. L., McLean, S., Kendra, P. E., Ploetz, R. C., & Pena, J. E. (2012). Effect of trap size, trap height and age of lure on sampling *Xyleborus glabratus* (Coleoptera: Curculionidae: Scolytinae), and its flight periodicity and seasonality. *Florida Entomologist*, 95(4):1003-1011.

Browne, F.G. (1961), The biology of Malayan Scolytidae and Platypodidae. *Malayan Forest Records*, 22, 1– 255.

Byers, J. A., Maoz, Y., Cohen, B., Golani, M., Fefer, D., & Levi-Zada, A. (2021). Protecting avocado trees from ambrosia beetles by repellents and mass trapping (push–pull): experiments and simulations. *Journal of Pest Science*, *94*(3), 991-1002.

Capecki, Z. (1963). *Perniphora robusta* Ruschka (Pteromalidae, Hymenoptera) and *Ipideurytoma spessivtsevi* Bouč. et Nov. (Eurytomidae, Hymenoptera) parasites of *Trypodendron lineatum* Oliv. (Scolytidae, Coleoptera) in Poland. *Ekol. Polska*, *11*, 303-308.

Carrillo, J. D., Dodge, C., Stouthamer, R., & Eskalen, A. (2020). Fungal symbionts of the polyphagous and Kuroshio shot hole borers (Coleoptera: Scolytinae, *Euwallacea spp.*) in California can support both ambrosia beetle systems on artificial media. *Symbiosis*, *80*(2), 155-168.

Clausen, C. P. (1940). *Entomophagous insects*. McGraw-Hill book Company, Incorporated.

Cook, J. M., & Crozier, R. H. (1995). Sex determination and population biology in the Hymenoptera. *Trends in Ecology & Evolution*, *10*(7), 281-286.

Cowan, D. P., & Stahlhut, J. K. (2004). Functionally reproductive diploid and haploid males in an inbreeding hymenopteran with complementary sex determination. *Proceedings of the National Academy of Sciences*, *101*(28), 10374-10379.

Dahlsten, D. L. (1982). Relationships between bark beetles and their natural enemies. Bark beetles in North American conifers. J. B Mitton, K. B Sturdgeon, ed. Ausin, University of Texas. 140–182.

Darling, D. C., & Roberts, H. (1999). Life history and larval morphology of Monacon (Hymenoptera: Perilampidae), parasitoids of ambrosia beetles (Coleoptera: Platypodidae). *Canadian Journal of Zoology*, *77*(11), 1768-1782.

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.

Eichhorn, O., & Graf, P. (1974). On some timber bark-beetles and their enemies. Anzeiger für Schadlingskunde, Pflanzen- und Umweltschutz, 47(9):129-135.

Escobar-Ramírez, S., Grass, I., Armbrecht, I., & Tscharntke, T. (2019). Biological control of the coffee berry borer: main natural enemies, control success, and landscape influence. *Biological control*, *136*, 103992.

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 Disease*, *97*(7), 938-951.

Fischer, M. (1954). Untersuchungen uber den kleinen Holzbohrer (*Xyleborinus saxeseni* Ratz). Pflanzenschutzberichte, *12*, 137-180.

Gordh, G. & Moczar, L. 1990. A catalog of the world Bethylidae (Hymenoptera: Aculeata). Memoirs of the American Entomological Institute. Gainesville, Florida American Entomological Institute Vol. 46 pp. 364.

Hall, T.A. (1999). BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41, 95-98.

Hedqvist, K. J. (1963). Die Feinde der Borkenkafer in Schweden I Erzwespe (Chalcidoidea)-Studia Forestalia Suecica, *11*, 1-176.

Herting, B. (1973). Coleoptera to Strepsiptera. *A catalogue of parasites and predators of terrestrial arthropods. Section A. Host or Prey/Enemy*, *3*(9).

Honsberger, D. N., & Wright, M. G. (2022). A new species of *Phymastichus* (Hymenoptera: Eulophidae: Tetrastichinae) parasitic on Xyleborus beetles (Coleoptera: Curculionidae: Scolytinae) in Hawai‘i, and aspects of its biology, life history, and behavior. *Zootaxa*, *5116*(1), 107-122.

Howard, R. W., & Pérez-Lachaud, G. (2002). Cuticular hydrocarbons of the ectoparasitic wasp *Cephalonomia hyalinipennis* (Hymenoptera: Bethylidae) and its alternative host, the stored product pest *Caulophilus oryzae* (Coleoptera: Curculionidae). *Archives of Insect Biochemistry and Physiology: Published in Collaboration with the Entomological Society of America*, *50*(2), 75-84.

Hulcr, J. & Stelinski, L. L. (2017). The ambrosia symbiosis: from evolutionary ecology to practical management. Annual Review of Entomology, 62: 285–303.

Inward, D. J. (2020). Three new species of ambrosia beetles established in Great Britain illustrate unresolved risks from imported wood. *Journal of Pest Science*, *93*(1), 117-126.

Jepson, F.P. (1920). Shot-hole borer of tea. *Tropical Agriculture*, *5*, 280-289.

Jervis, M. A., Kidd, N. A. C., Fitton, M. G., Huddleston, T., & Dawah, H. A. (1993). Flower-visiting by hymenopteran parasitoids. *Journal of natural history*, *27*(1), 67-105.

Jiang, Z., Kajimura, H. (2020). Earwig preying on ambrosia beetle: Evaluating predatory process and prey preference. Journal of Applied Entomology, *144*, 743–750.

Kalshoven, L. G. E. (1959). New cases of synonymy in Indomalayan scolytids. *Entomologische Berichten*, *19*(5), 93-97.

Kalshoven, L. G. E. (1960). Observations on the parasites of *Xyleborus* twig borers in Java. Entomologische Berichten, *20*, 259-262.

Kenis, M., Wermelinger, B., Gregoire, J. C. (2004). Research on Parasitoids and Predators of Scolytidae – A Review. Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis: 237-290.

Kirkendall, L. R., Kent, D. S., & Raffa, K. F. (1997). 9· Interactions among males, females and offspring in bark and ambrosia beetles: the significance of living in tunnels for the. *The Evolution of Social Behaviour in Insects and Arachnids*, 181.

Kirkendall, L. R., Biedermann, P. H., & Jordal, B. H. (2015). Evolution and diversity of bark and ambrosia beetles. In *Bark beetles* (pp. 85-156). Academic Press.

Kumar, S., Stecher, G., Li, M., Knyaz C., Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution, 35*, 1547-1549.

La Salle, J. (1990). A new genus and species of Tetrastichinae (Hymenoptera: Eulophidae) parasitic on the coffee berry borer, *Hypothenemus hampei* (Ferrari)(Coleoptera: Scolytidae). *Bulletin of Entomological Research*, *80*(1), 7-10.

La Salle, J. (1995). A new species of *Phymastichus* (Hymenoptera: Eulophidae) parasitic on adult *Xyleborus perforans* (Coleoptera: Scolytidae) on macadamia trees in Hawaii. *Proceedings of the Hawaiian Entomological Society*, *32*, 95-101.

Lauzière, I., Brodeur, J., & Pérez-Lachaud, G. (2001). Host stage selection and suitability in *Cephalonomia stephanoderis* Betrem (Hymenoptera: Bethylidae), a parasitoid of the coffee berry borer. *Biological control*, *21*(2), 128-133.

Librado, P., & Rozas, J. (2009). DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics, 25*, 1451-1452.

Lona, I. D., Miller III, D. G., Hatfield, C. A., Rosecrance, R. C., Nelson, L. J., Audley, J. P., Siefker, M. A., Chen, Y., & Seybold, S. J. (2020). Host selection behavior mediated by differential landing rates of the walnut twig beetle, *Pityophthorus juglandis*, and associated subcortical insect species, on two western North American walnut species, *Juglans californica* and *J. major*. *Entomologia Experimentalis et Applicata*, *168*(3), 240-258.

MacLean, D. B., & Giese, G. L. (1967). The life history of the ambrosia beetle *Xyloterinus politus* (Coleoptera: Scolytidae). *Canada Entomology*, *99*, 285-299.

Marsh, P. M. (1979). Aphidiidae. *Catalog of Hymenoptera in America north of Mexico, 1*, 295-313.

Mathews, P. L., & Stephen, F. M. (1997). Effect of artificial diet on longevity of adult parasitoids of Dendroctonus frontalis (Coleoptera: Scolytidae). *Environmental entomology*, *26*(4), 961-965.

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*(3), 235-238.

Mitchell, A., & Maddox, C. (2010). Bark beetles (Coleoptera: Curculionidae: Scolytinae) of importance to the Australian macadamia industry: an integrative taxonomic approach to species diagnostics. *Australian Journal of Entomology*, *49*(2), 104-113.

Muraleedharan, N., Selvasundaram, R., & Radhakrishnan, B. (1988). Natural enemies of certain tea pests occurring in southern India. *International Journal of Tropical Insect Science*, *9*(5), 647-654.

Noyes, J. S. (2001). Interactive Catalogue of World Chalcidoidea 2001. CD Rom. Vancouver, Canada: Taxapad.

Noyes, J. S. (2003). Universal Chalcidoidea Database. World Wide Web at: www.nhm.ac.uk/entomology/chalcidoids/about.html

Peer, K., & Taborsky, M. (2004). Female ambrosia beetles adjust their offspring sex ratio according to outbreeding opportunities for their sons. *Journal of evolutionary biology*, *17*(2), 257-264.

Le Pelley, R. H. (1968). *Pests of coffee*. Longmans, Green and Co. Ltd., London and Harlow. 590 pp.

Paap, T., De Beer, Z. W., Migliorini, D., Nel, W. J., & 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., Weihman, S. W., McLean, S., Cave, R. D., Carrillo, D., Duncan, R. E., Evans, G., Krauth, S., Thomas, M. C., Lu, S. S., Kendra, P. E., & Roda, A. L. (2015). Predators and parasitoids associated with Scolytinae in *Persea* species (Laurales: Lauraceae) and other Lauraceae in Florida and Taiwan. *Florida Entomologist*, *98*(3), 903-910.

Pérez-Lachaud, G., & Hardy, I. C. W. (1999). Reproductive Biology of *Cephalonomia hyalinipennis* (Hymenoptera: Bethylidae), a Native Parasitoid of the Coffee Berry Borer, *Hypothenemus hampei* (Coleoptera: Scolytidae), in Chiapas, Mexico. *Biological Control*, *14*(3), 152–158.

Petters, R. M., & Mettus, R. V. (1980). Decreased diploid male viability in the parasitic wasp, *Bracon hebetor*. *Journal of Heredity*, *71*(5), 353-356.

Portilla, M. (1999). Mass rearing technique for *Cephalonomia stephanoderis* (Hymenoptera: Bethylidae) on *Hypothenemus hampei* (Coleoptera: Scolytidae) developed using Cenibroca artificial diet. *Revista Colombiana de Entomología*, *25*(1), 57-66.

Prebble, M. L., Graham, K. (1957). Studies of attack by ambrosia beetles in softwood logs on Vancouver Island, British Columbia. *Forest Science*, *3*(1), 90-112.

Qin, Y. G., Zhou, Q. S., Yu, F., Wang, X. B., Wei, J. F., Zhu, C. D., Zhang, Y. Z., & Vogler, A. P. (2018). Host specificity of parasitoids (Encyrtidae) toward armored scale insects (Diaspididae): Untangling the effect of cryptic species on quantitative food webs. *Ecology and evolution*, *8*(16), 7879–7893.

Quicke, D. L. J. (2015). *The braconid and ichneumonid parasitoid wasps: Biology, systematics, evolution and ecology*. John Wiley & Sons, Incorporated, 43.

Rabaglia, R. J., Dole, S. A., & 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.

Rahman, M. M., Islam, W., & Ahmed, K. N. (2008). Fertility life tables of *Plastanoxus westwoodi* (Kieffer) (Hymen optera: Bethylidae) on *Cryptolestes pusillus* (Schon.) (Coleoptera: Cucujidae). *Journal of Biological Science*, *16*, 25–28.

Ratnasingham, S., & Hebert, P. D. (2007). bold: The Barcode of Life Data System (http://www.barcodinglife.org). Molecular ecology notes, *7*(3), 355–364.

Rivera, M. J., Martini, X., Conover, D., Mafra-Neto, A., Carrillo, D., & Stelinski, L. L. (2020). Evaluation of semiochemical based push-pull strategy for population suppression of ambrosia beetle vectors of laurel wilt disease in avocado. *Scientific reports*, *10*(1), 1-12.

Roberts, H. (1968). Notes on the biology of ambrosia beetles of the genus *Trachyostus* Schedl (Coleoptera: Platypodidae) in West Africa. *Bulletin of Entomological Research*, *58*(2), 325-352.

Rugman-Jones, P. F., Au, M., Ebrahimi, V., Eskalen, A., Gillett, C. P., Honsberger, D., Husein, D., Wright, M. G., Yousuf, F., & Stouthamer, R. (2020). One becomes two: second species of the *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae) species complex is established on two Hawaiian Islands. *PeerJ*, *8*, e9987.

Shaw, S. R., & Berry, J. A. (2005). Two new *Cryptoxilos* species (Hymenoptera: Braconidae: Euphorinae) from New Zealand and Fiji parasitising adult scolytinae (coleoptera). *Invertebrate Systematics*, *19*(5), 371-381.

Seitner, M. & Notzl, P. (1925). *Pityophthorus henscheli* Seitner und sein Parasit *Cosmophorushenscheli* Ruschka. *Zeitschrift fu¨r angewandte Entomologie*, *11*, 187–195.

Spennemann, D. H. (2019). Biology, ecology and distribution of the Date Stone Beetle, *Coccotrypes dactyliperda* (Coleoptera: Curculionidae). *Zoology in the Middle East*, *65*(2), 163-182.

Stecher, G., Tamura, K., Kumar, S. (2020). Molecular Evolutionary Genetics Analysis (MEGA) for macOS. Molecular Biology and Evolution (https://doi.org/10.1093/molbev/msz312).

Stigenberg, J., Boring, C. A., & Ronquist, F. (2015). Phylogeny of the parasitic wasp subfamily Euphorinae (Braconidae) and evolution of its host preferences. *Systematic Entomology*, *40*(3), 570-591.

Stouthamer, R., Luck, R. F., & Werren, J. H. (1992). Genetics of sex determination and the improvement of biological control using parasitoids. *Environmental Entomology*, *21*(3), 427-435.

Stouthamer, R., Breeuwer, J. A., & Hurst, G. D. (1999). *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annual Reviews in Microbiology*, *53*(1), 71-102.

Stouthamer, R., Rugman-Jones, P., Thu, P. Q., Eskalen, A., Thibault, T., Hulcr, J., Wang, L., Jordal, B. H., Chen, C., Cooperband, M., Ling, C., Kamata, N., Lu, S., Masuya, H., Mendel, Z., Rabaglia, R., Sanguansub, S., HsinHui, S., Sittichaya, W., & Zong, S. (2017). Tracing the origin of a cryptic invader: phylogeography of the *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae) species complex. *Agricultural and Forest Entomology*, *19*(4), 366-375.

Swezey, O. H. (1941). Observations on insect pests in Samoa which are not yet known to occur in Hawaii. Hawaii. Plrs' Rec. *45*, 25–38.

Truett, G. E., Heeger, P., Mynatt, R. L., Truett, A. A., Walker, J. A., & Warman, M. L. (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques*, *29*(1), 52-54.

Vega, F. E., Infante, F., Castillo, A., & Jaramillo, J. (2009). The coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae): a short review, with recent findings and future research directions.

Villagra, C. A., Pinto, C. F., Penna, M., & Niemeyer, H. M. (2011). Male wing fanning by the aphid parasitoid *Aphidius ervi* (Hymenoptera: Braconidae) produces a courtship song. *Bulletin of entomological research*, *101*(5), 573-579.

Vinson, S. B. (1972). Courtship behavior and evidence for a sex pheromone in the parasitoid *Campoletis sonore*nsis (Hymenoptera: Ichneumonidae). *Environmental Entomology*, *1*(4), 409-414.

Wang, Y. P., Shi, M., Chen, X. X., He, J. H. (2009). The genus *Atanycolus* Foerster (Hymenoptera, Braconidae, Braconinae) in China, with description of one new species. *ZooKeys, 27*, 31-41.

Wood, S. L. (1982). The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. *The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph.*

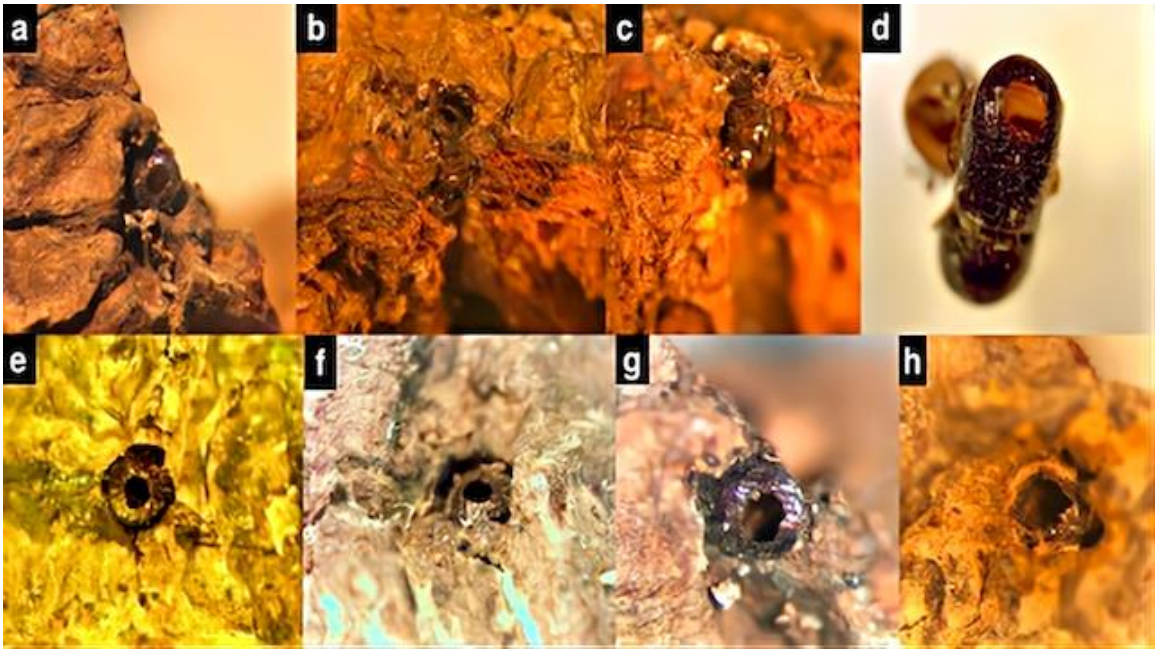

Figure 1.1 *E. fornicatus* beetle carcasses with emergence holes located on elytral declivity found on imported avocado logs and experimental castor bean logs with records of *Phymastichus* parasitoid emergence. **a-c:** Bark was removed from one side to display short gallery of beetle, later molecularly identified as *E. kuroshio*. **e-h:** *E. fornicatus* carcasses from castor bean experimental logs. **d:** Intact *E. fornicatus* beetle removed from wood material.

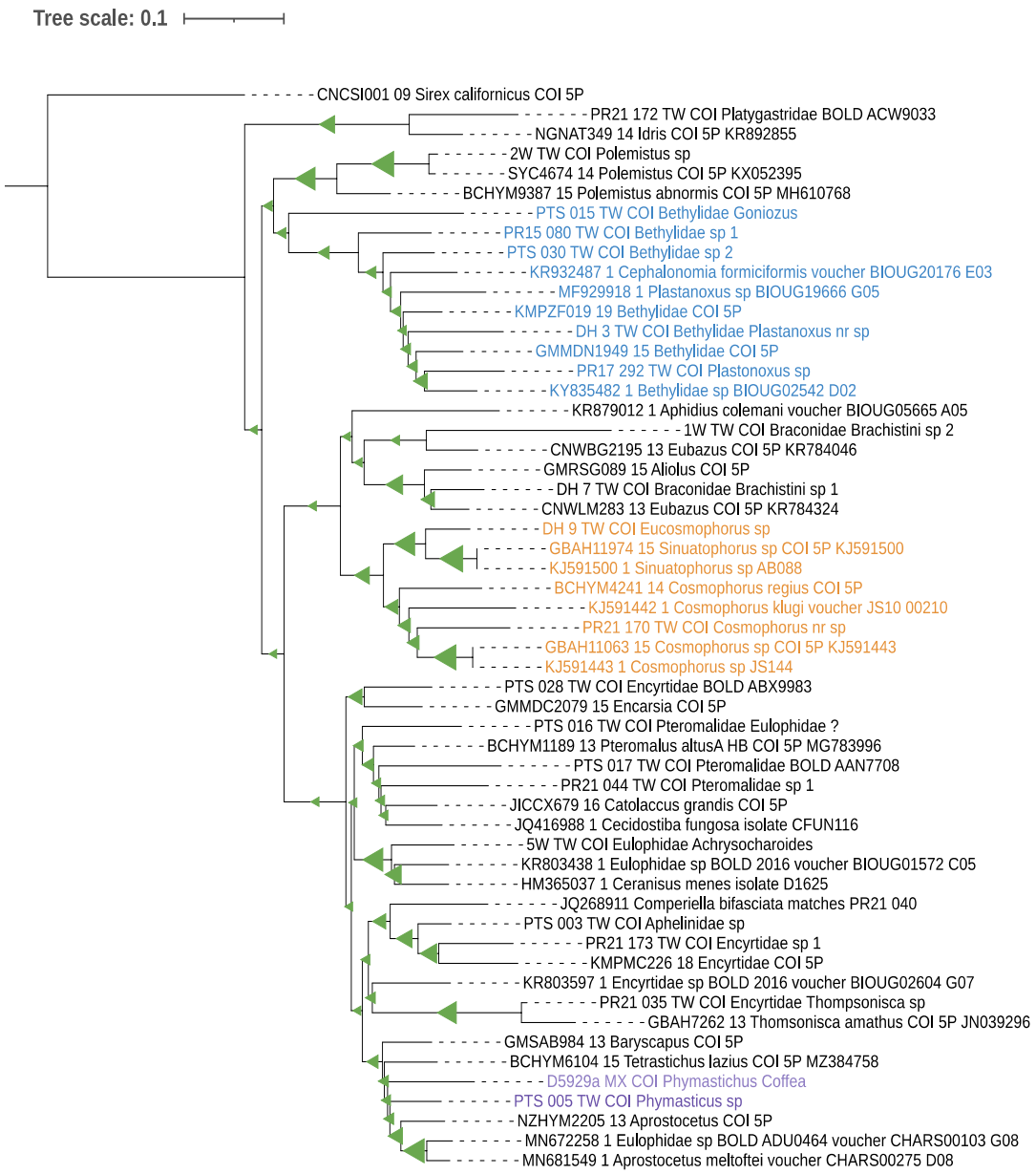

Figure 1.2. Taxonomic placement of parasitoids recovered from SHB-infested wood. Maximum likelihood tree inferred using the General Time Reversible model to estimate evolutionary relationships among 21 haplotypes (this study) and 33 closely matching accessions retrieved from GenBank and BOLD. The bootstrap consensus tree inferred from 500 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were not collapsed. Highlighted sections represent the three parasitoid species of interest along with accessions of related species in that group.

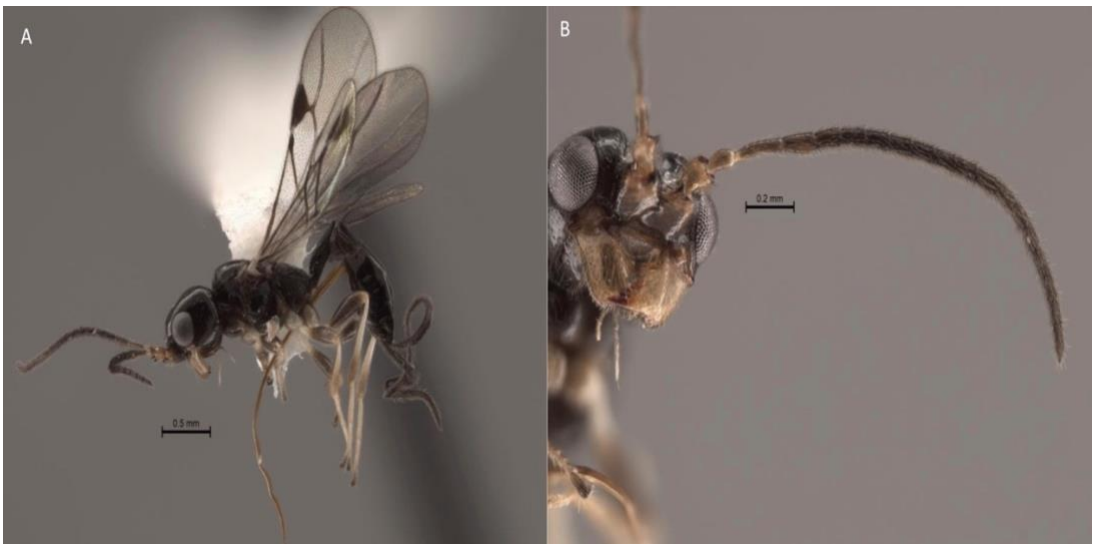

Figure 1.3. (A): Habitus image of female *Eucosmophorus sp.* with ovipositor unsheathed. **(B):** Female *Eucosmophorus sp.* headshot with closer view of mandibles.

Figure 1.4. Habitus image of male *Eucosmophorus sp*.

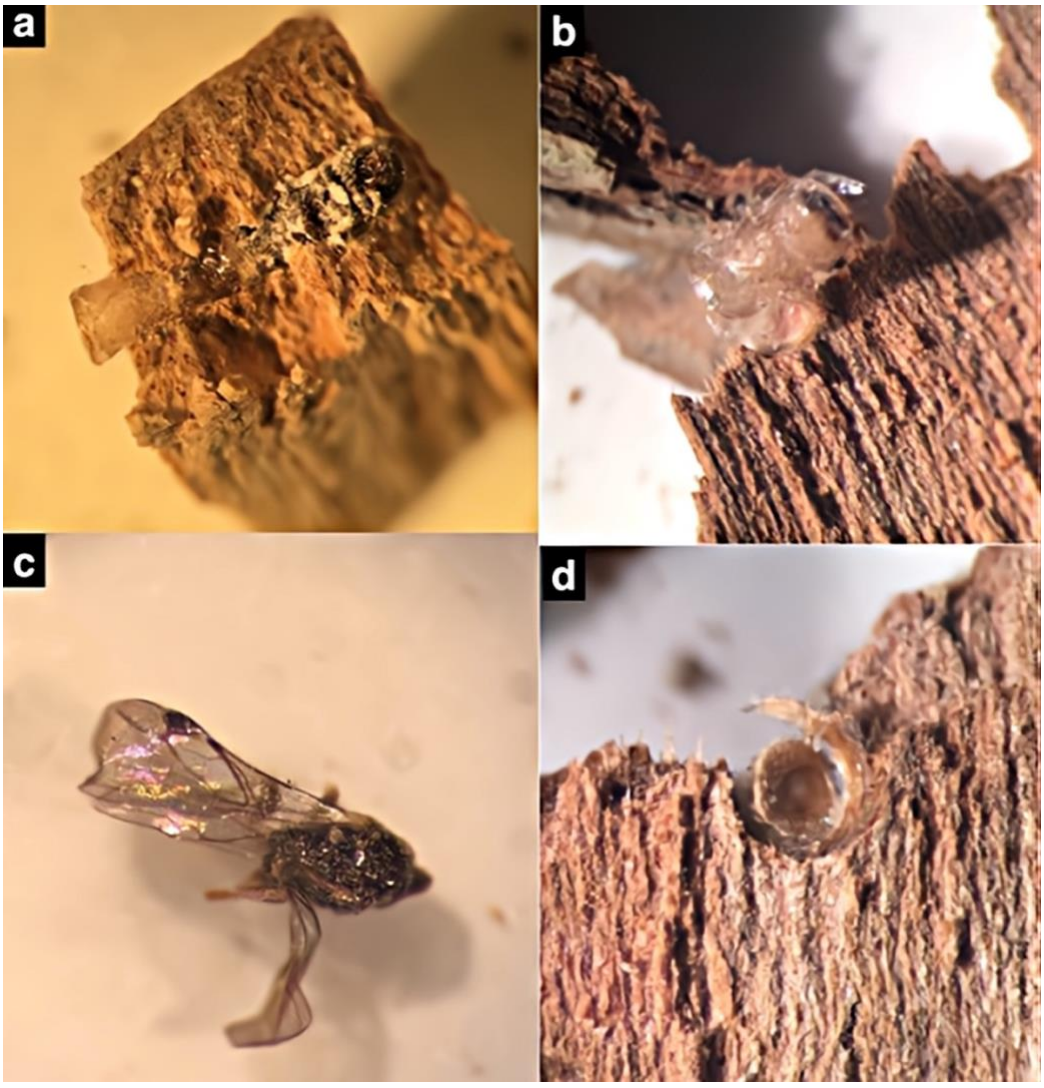

Figure 1.5. Compiled images taken from dissected *E. fornicatus* lab-infested logs that have only been introduced to the braconid *Eucosmophorus*. **c:** A desiccated *Eucosmophorus* wasp that assumably encountered a molting accident was removed from **(a)** a dry, film-like cocoon extended away from the abdomen of an *E. fornicatus* beetle carcass. **b and d**: Additional images of damaged cocoons.

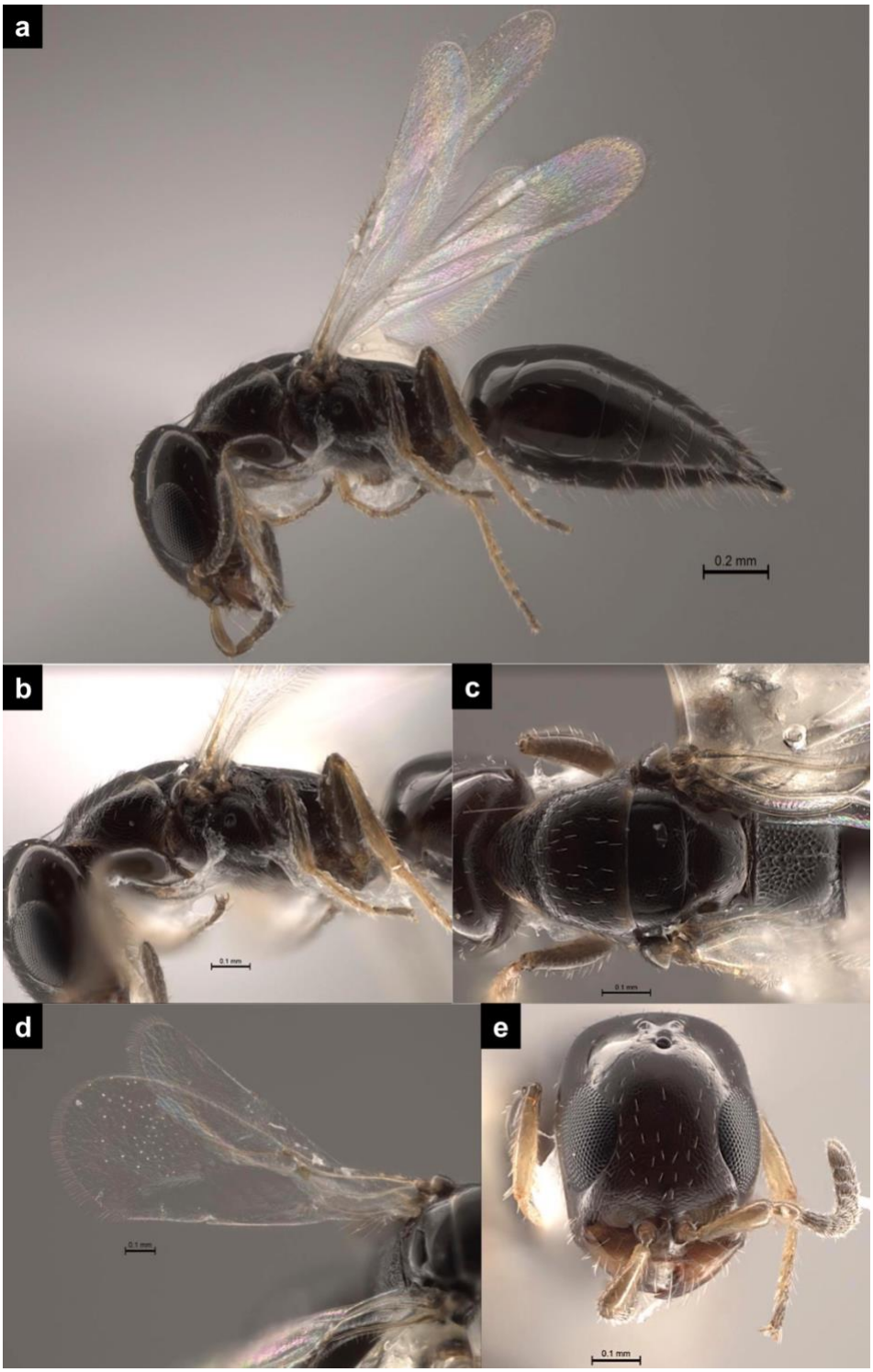

Figure 1.6. **(a)** Habitus, **(b)** mesosoma lateral, **(c)** mesosoma dorsal, **(d)** wing, and **(e)** face images taken of a *Plastanoxus* adult parasitoid that emerged from shothole borer-infested avocado logs imported from Taiwan.

Figure 1.7. Female *Phymastichus sp*. that emerged from native *E. fornicatus species complex*-infested avocado bolts.

Figure 1.8. Habitus shot of male *Phymastichus sp.* **(left)** along with a close up on the face to exhibit the absence of the vestral sensory plaque **(right)**.

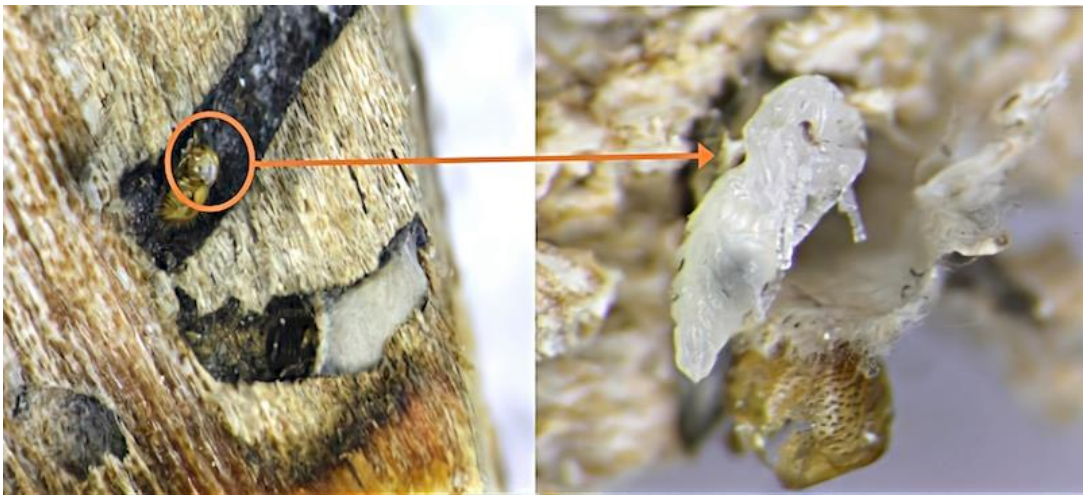

Figure 1.9. (left): Two *E. fornicatus* beetles parasitized by an unknown parasitoid with one cocoon intact beside a beetle's head. Pupa **(right)** of a parasitoid wasp extracted from detached cocoon later identified as a bethylid.

Table 1.1. Parasitoids either mentioned in literature or directly observed to be associated with an ambrosia beetle in the subfamilies Scolytinae and Platypodinae.

Main references[1]	*Crossotarsus barbatus*	*Xyleborus affinis*	*Xyleborus dispar*	*Xyleoborus ferrugineus*	*Xyleborus perforans*	*Xyleborinus saxesenii*	*Xylosandrus morigerus*	*Xyloterinus politus*	*Xylosandrus compactus*	*Trypodendron domesticum*	*Trypodendron lineatum*
Bethylidae											
Prorops nasuta	.	.	.	.	.	.	.	.	l, n	.	.
Unknown Bethylidae sp.	.	.	.	.	.	.	l	.	l	.	.
Braconidae											
Atanycolus lindemani	.	.	.	.	.	.	.	s	.	.	.
Blacus fuscipes	.	.	.	.	.	g	.	.	.	.	.
Cosmophorus regius	.	.	.	.	.	.	.	.	.	.	p
Eulophidae											
Phymastichus holoholo	.	j	.	j	j	.	.	.	.	.	.
Phymastichus xylebori	.	.	.	.	m	.	.	.	.	.	.
Tetrastichus xyleborорum	.	.	.	.	.	.	k	.	l	.	.
Tetrastichus n. sp. xyleborорum	.	.	.	.	.	.	.	.	e	.	.
Eupelmidae											
Unkknown Eupelmidae sp.	.	.	.	.	.	.	.	.	a	.	.
Eurytomidae											
Eurytoma afra	.	.	f	.	.	p	.	.	.	p	f, q
Eurytoma morio	.	.	b, h, p	.	.	.	.	.	.	.	.
Ipideurytoma sp.	.	.	.	.	.	.	.	o	.	.	.
Perilampidae											
Monacon roberstsi	d	.	.	.	.	.	.	.	.	.	.
Pteromalidae											
Habritys brevicornis	.	.	f, r	.	.	.	.	.	.	h	.
Perniphora robusta	.	.	b, c, h, f	.	.	b, c, h, f	.	.	.	b, c, h, k, p	i
Platygerrhus affinis	.	.	.	.	.	.	.	.	.	.	h
Vrestovia querci	.	.	f	.	.	.	.	.	.	.	.

[1] References: a: Balakrihnan et al. (1989); b: Balazy (1963); c: Capecki (1963); d: Darling and Roberts (1999); e: Dhanam et. al (1992); f: Eichhorn and Graf (1974); g: Fischer (1954); h: Hedqvist (1963); i: Herting (1973); j: Honsberger and Wright, (2022); k: Kalshoven (1960); l: Le Pelley (1968); m: La Salle (1995); n: Lavabre (1962); o: MacLean and Giese (1967); p: Noyes (2001); q: Prebble and Graham (1957); r: Thomoson (1943); s: Wang et al. (2009).

Table 1.2. COI and 28S identification of parasitoid wasps associated with avocado and castor bean wood cuttings infested with beetles of the *Euwallacea fornicatus* cryptic species complex from Taiwan. Tentative ID of emerged parasitoids was inferred from the closest % ID match in the Barcode of Life Database (BOLD) and GenBank, which included a query cover greater than 88%. GenBank accessions of deposited sequences are listed with the appropriate parasitoid sample. The identification of specimens with sequence IDs with lower than 90% should be regarded as very tentative.

Families of Emerged Parasitoids	Representative Sample ID	GenBank Accession		Closest 28S GenBank Accession	% ID	Closest COI GenBank Accession	% ID	BOLD Barcode Bin Number (BIN)	Similarity %	Tentative ID
		COI	28S							
Aphelinidae	PTS-003	OP933733	OP924516	---	---	KF778461	90.54%	---	---	Aphelinidae sp.
Bethylidae	PTS-071	---	OP924517	JX413931	98.73%	---	---	---	---	Bethylidae: *Allobethylus* sp.
	PTS-030	OP933734	OP924518	KC762949	96.46%	MG445788	90.44%	ADE7970	99.52%	Bethylidae sp.
	PR15-080	OP933735	OP924519	KC762949	89.88%	---	---	---	---	Bethylidae sp.
	DH-3	OP933736	---	---	---	---	---	ADV1646	89.62%	Bethylidae sp.
	PTS-015	OP933737	OP924520	KU753521	95.21%	---	---	Private	98.91%	Bethylidae sp.
	PR17-292	OP933738	OP924521	KC762949	93.92%	---	---	ACU2045	90.03%	*Plastanoxus* sp.*
	PR20-586	---	OP924522	KC762949	97.24%	---	---	---	---	*Plastanoxus sp.*
Braconidae	DH-1W	OP933739	OP924523	JF979699	92.93	---	---	---	---	Brachistinae sp.
	DH-07	OP933740	---	---	---	KR782535	93.43%	AAG1266	93.33%	Braconidae sp.
	PR21-170	OP933741	OP924524	KJ591230	96%	---	---	---	---	Euphorinae: *Cosmophorus* sp.
	PR17-040	OP933742	OP924525	KJ591296	98.80%	KJ591500	92.67%	Private ACV9738	93.24% 92.63%	*Eucosmophorus* sp.*
Crabronidae	DH-2W	OP933743	OP924526	OW121816	90.65%	KX052395	99.85%	ACN4161	99.85%	*Polemistus* sp.
Encyrtidae	PR21-040	OP933744	OP924527	AY599317	99.82%	JQ268911	100%	ACB8168	100%	*Comperiella bifasciata*
	PR21-173	OP933745	OP924528	---	---	---	---	ADZ5355	88.38%	Encyrtidae sp.
	DH-4W	---	OP924529	KX868554	84.05%	---	---	---	---	Encyrtidae sp.
	PTS-028	OP933746	OP924530	AY599321	91.35%	KY831595	99.85%	ABX9983	99.84%	Encyrtidae sp.
	PR21-035	OP933747	OP924531	KX091706	96.67%	KX091484	96.24%	Private	96.82%	*Thompsonisca sp.*
Eulophidae	PR21-033	---	OP924532	AY580329	98.38%	---	---	---	---	*Aprostocetus* sp.
	DH-5W	OP933748	OP924533	AY771701	97.10%	MZ631606	90.72%	---	---	Eulophidae sp. **
	PR17-299	OP933749	OP924534	AY580329	96.54%	JN293500	90.63%	ADF5297	90.40%	*Phymastichus* sp.*
Platygastridae	PR21-172	OP933750	OP924535	MF583399	94.86%	---	---	ACW9033	99.12%	Platygastridae sp.
	PR21-037	---	OP924536	DQ888477	84.73%	---	---	---	---	Platygastridae sp.
Pteromalidae	PTS-017	OP933751	OP924537	JN623812	94.96%	HQ929645	100%	AAN7708	99.38%	Pteromalidae sp.
Torymidae	PR21-044	OP933752	OP924538	MF952357	95.44%	---	---	---	---	Torymidae sp.*
Inconclusive	PTS-016	OP933753	OP924539	HE793293	95.91%	---	---	Private	91.24%	Eulophidae sp. / Pteromalidae sp. **

* Morphologically identified to genus level.

** Inconclusive; close % match to more than one genus/family.

Table 1.3. Emergence data of three parasitoid species collected from imported SHB-infested wood and successfully reared on PSHB-only infested wood in the lab. P1 (parental generation) accounts for the number of parasitoids that emerged directly from wood imported from Taiwan. Each parasitoid species produced at least one filial generation (F1). Parasitoid emergence numbers in each filial generation (F1-F3) were documented from lab-controlled PSHB-infested avocado and castor bean logs.

Shipments dates	# of parasitoid emergence															
	Braconidae (*Eucosmophorus sp.)*						Eulophidae (*Phymastichus sp.)*								Bethylidae *(Plastanoxus sp.)*	
	P1		F1		F2		P1		F1		F2		F3		P1	F1
	f	*m*	*f*	*m*	*f*	*m*	*f*	*m*	*f*	*m*	*f*	*m*	*f*	*m*	*u*	*u*
Jan-2017	46	46	41	76	-		7	7	-		-		-		6	-
May-2017	16	16	9	12	4	16	13	11	8	8	5	5	3	2	33	7
Apr-2018	17	19	-		-		-		-		-		-		-	-
Jan-2019	21	28	-		-		-		-		-		-		8	-
Nov-2019	41	51	-	5	-		-		-		-		-		-	-

Chapter 2: Uncovering cryptic parasitoid-host relationships through molecular gut content analysis

Abstract

In recent years, biological control efforts have focused on the discovery, identification, and mass-rearing of natural enemies associated with the *Euwallacea fornicatus* species complex, in particular, the polyphagous shot hole borer (PSHB), *E. fornicatus sensu stricto*. PSHB is an invasive ambrosia beetle that has caused significant damage in numerous native and ornamental trees, as well as agricultural crops, in southern California and other regions, worldwide. Multi-year collection of parasitoids emerging from native shothole borer-infested wood, lead to the identification of several potential natural enemies, of which, one commonly encountered species, and six less common species, belonged to the flat wasp family, Bethylidae. In the PSHB system, the acts of parasitism and parasitoid emergence are impossible to observe directly since they occur within the beetle gallery, inside the woody tissue of an infested tree. Thus, in an attempt to confirm the parasitic link between PSHB and each parasitoid, we designed a probe-based qPCR assay to detect remnants of PSHB DNA in the gut of the parasitoids. Detection limits of the assay were determined using 10-fold serial dilutions of PSHB DNA in that of a "surrogate" parasitoid species, *Tamarixia radiata*, a parasitoid of the Asian citrus psyllid. Among the parasitoids that emerged from the native infested logs, only two species consistently showed evidence of having consumed PSHB, namely the Braconids, *Eucosmophorus* sp. and *Cosmophorus* sp. None of the bethylids consistently tested positive, not even a *Phymastichus* sp. that, through laboratory rearing, has already

been shown to parasitize PSHB. Reasons for this discrepancy and the implications of our findings are discussed.

Introduction

Pest management of the *Euwallacea fornicatus* species complex (Coleoptera: Curculionidae: Scolytinae) has proven to be largely difficult due to their cryptic lifestyle. Generally, a single female can establish a colony, with or without prior mating, as a result of haplodiploid reproduction and sib-mating; a commonly observed trait of ambrosia beetles in the Xyleborini tribe (Browne, 1961; Beaver, 1988). Management is further complicated by the symbiotic relationship with ambrosia fungi carried by the beetles in vestigial pockets, mycangia, and utilized as their main food resource across all stages of development. Conventional methods of control, i.e. topical and systemic pesticide, may be achievable at low levels of infestation, but are costly and require continuous application and frequent monitoring (Mayorquin et al., 2014; Eatough Jones and Paine, 2018). Thus, we directed our investigation toward biological control.

Biological control requires the accurate recognition of natural enemy-host/prey interactions (Greathead, 1997). This is not typically an issue since most natural enemy host interactions can be directly observed. Provided that the interacting parties are accurately identified, this can lead to a straightforward progression from finding the natural enemy in the native range of the host, to mass rearing, and host-natural enemy interaction studies. However, progressing through these steps is more complicated if parasitized hosts/prey lead a hidden lifestyle, and share their habitat with other potential prey/host species that may also be attacked by the natural enemies. We encountered this situation when trying to identify the host-parasitoid relationships for beetles of the *E. fornicatus* species complex. Females of these species create galleries inside the branches

of host trees, where they produce their offspring. Apart from a very brief dispersal phase (recently eclosed adult females only), they spend their entire lives hidden within the tree. Furthermore, tree branches that are infested with *E. fornicatus* often also contain other insects (Pena et al., 2015). Establishing explicit links between parasitoids and members of the *E. fornicatus* species complex is therefore fundamental to correctly identifying potential biological control agents for further assessment.

How then to identify the natural enemies emerging from such logs as being parasitoids of specifically beetles of the *E. fornicatus* complex? One solution is to rear emerged natural enemies on laboratory colonies of only *E. fornicatus* beetles. Such an approach was successful in Chapter 1 with two of the parasitoids emerging from field collected logs from the native range of the beetles: an as yet undescribed *Eucosmophorus sp.* (Hymenoptera: Braconidae) and an undescribed *Phymastichus sp.* (Hymenoptera: Eulophidae). However, this rearing approach proved to be problematic for several species in the family Bethylidae that also emerged from those logs. Therefore, as an alternative method to establish host parasitoid relationships, we explored the possibility of detecting host DNA within parasitoids that emerged from field collected logs. Although it is generally assumed that DNA consumed by a parasitoid as a larva is voided from the parasitoid gut during metamorphosis (in the form of meconium), in parasitoids of several lepidopteran hosts, it has been shown that the DNA of the larval-host can in fact still be detected in the gut of the adult parasitoids (Rougerie et al., 2011; Wirta et al., 2014; Mutanen et al., 2022). Furthermore, in some taxa (e.g., Bethylidae), adult parasitoids may also contribute towards host mortality, and therefore acquire host DNA, by host feeding.

In one of the best studied host parasitoid interactions involving ambrosia beetles, the coffee berry borer, *Hypothenemus hampei*, (Coleoptera: Curculionidae) is parasitized by two Bethylid species, *Prorops nasuta* and *Cephalonomia stephanoderis* (Hymenoptera: Bethylidae) (Abraham, 1990; Escobar-Ramírez et al., 2016), both of which also frequently feed as adults, on the larval stages of their hosts.

In this study, we developed a molecular assay to detect trace amounts of beetle DNA within the DNA extracted from adult parasitoid wasps that emerged from wood, infested with members of the *E. fornicatus* species complex, imported from Taiwan. The objectives of this study were to 1) develop and optimize a fluorescent probe-based real-time PCR assay to detect trace amounts of DNA from beetles belonging to the *E. fornicatus* cryptic species complex, and 2) elucidate cryptic links of additional parasitoids associated with the *E. fornicatus* cryptic species complex and further expand the list of candidate biological control agents.

Materials and Methods

Development of a fluorescent probe-based molecular assay to detect trace amounts of shothole borer DNA within parasitoids

Since four different SHB taxa co-occur in Taiwan (PSHB, KSHB, TSHB, and H22; Stouthamer et al., 2017; Liu et al., 2022), parasitoids may potentially develop on one or more of these hosts. In this study we were not concerned with identifying which particular species, but rather, if a parasitoid utilized any of those four. Thus, we designed an assay targeting the conserved 28S rRNA. Existing sequences of the 28S-D2 region of

PSHB, KSHB, TSHB, and H22 (GenBank accessions MT822791, KU727079, MT822792, and KU727084, respectively) were aligned manually using BioEdit 7.0.5.3, (Hall, 1999). The software program Primer3 (Untergasser et al. 2012) was used, via the NCBI Primer-BLAST tool, to design three potential assays, each targeting a slightly different conserved region of 28S-D2 **(Figure 2.1)**. The specificity of each primer/probe combination to only amplify SHB DNA was also evaluated using Primer-BLAST to check for possible mis-priming with the 28S-D2 region of each of the parasitoid taxa identified in Chapter 1 (GenBank accession #s: OP924516-OP924539).

As a first step towards assay development, the primers for each of the three assays were obtained from Integrated DNA Technologies, Inc. (IDT, Coralville, IA, USA) and conventional gradient-PCRs were run in order to determine what annealing temperature worked for each primer set. All primer sets worked well at an annealing temperature of 61°C. We then used dye-based real-time PCR (qPCR) to determine which primer set yielded the greatest amplification across all four SHB taxa. Reactions were performed in 20 µL volumes containing 1X HOT FIREPol® EvaGreen® qPCR Mix (Mango Biotechnology LLC, Mountain View, CA, USA), 200 nM each forward and reverse primers, and 2 µL of template DNA. The qPCR was performed on a Rotor-Gene Q (QIAGEN) with an initial cycle of 95ºC for 15 min, followed by 40 cycles of 95ºC for 20 s, 61ºC for 20 s, and 72ºC for 30 s, with fluorescence being measured on the green channel (excitation 470 nm / detection 510 nm) at the end of each 72ºC extension step. Primer set 3 was judged to produce the best amplification (not shown), and to continue

with the development of assay 3, the respective double-quenched PrimeTime™ fluorescent hydrolysis probe (reporter dye = 6-FAM) was obtained from IDT.

The amplification efficiency of the newly designed primer/probe set was examined in real-time PCR (qPCR) using "bulk" PSHB DNA collected by pooling 100 µL from each of 20 individual HotSHOT extractions. Pooled DNA was used in an effort to ensure that the starting concentration represented that of an average individual PSHB. The pooled DNA was mixed by pipetting the entire volume up and down 10 times, and then used to create a series of 10-fold dilutions (up to 10^{-6}) of the beetle DNA. Rather than diluting the PSHB DNA in water, which is not biologically realistic, the diluent used was instead DNA extracted from a "surrogate" parasitoid. As a parasitoid of the Asian citrus psyllid, *Tamarixia radiata* was chosen as the surrogate because of its obvious non-association with *Euwallacea* spp., and because of its availability in our laboratory. Thus, the diluent was created by pooling multiple (n=20) individual HotSHOT DNA extractions of *T. radiata*, each performed in a volume of 80 µL, resulting in an overall volume of *T. radiata* DNA equating ~1.5 mL. This was sufficient to produce a series of standard dilutions, each of 200 µL, plus sufficient pure *T. radiata* DNA to use as a negative control throughout the study (see below). The dilution series was initiated by adding 20 µL of undiluted PSHB DNA to 180 µL of undiluted *T. radiata* DNA. This 1:10 dilution was mixed by pipetting the entire volume up and down 10 times, and then used to make the next dilution in the series; each time mixing 20 µL of the previous dilution with 180 µL of undiluted *T. radiata* DNA. Amplification efficiency was tested in 20 µL reactions containing 1X HOT FIREPol® Probe qPCR Mix Plus (Mango Biotechnology

LLC, Mountain View, CA, USA), 200 nM each forward and reverse primers, 80 nM fluorescent probe, and 2 μL of template DNA. The qPCR was performed on a Rotor-Gene Q (QIAGEN) with an initial cycle of 95°C for 15 min (to activate the polymerase), followed by 40 cycles of 95°C for 20 s, 61°C for 20 s, and 72°C for 30 s.

Each dilution was tested in three replicate reactions. Early qPCR optimization runs included two different negative controls (in duplicates). The first negative control contained unadulterated *T. radiata* DNA (to rule out non-specific amplification), and the second contained no-template at all (to ensure the reagents used in the assay were free from contamination). Subsequently, no-template controls were used for the remaining runs after *T. radiata* proved to not amplify. Fluorescence was recorded on the green channel (excitation 470 nm / detection 510 nm) at 72°C, at the end of each cycle, and Auto-Gain optimization was performed on tube #1 prior to the first acquisition. Post-qPCR, Ct values and amplification efficiency were calculated using the Quantitation analysis module in the integrated Rotor-Gene Q software package v.2.3.4 (Build 3) with Dynamic Tube Normalization and Noise Slope Correction activated. Outlier removal was set at 2% and the threshold of amplicon detection was determined using the Auto Find Threshold function.

Parasitoid sample collection

Sampling of parasitoids took place in 2017 to 2021, as part of a project seeking to rear natural enemies of the *E. fornicatus* species complex (Husein et al., in review). Wood, infested with *Euwallacea* spp., was imported from Taiwan (part of the native range of the beetle complex) to the Insectary and Quarantine (I&Q) facility at the

University of California, Riverside (UCR), California, USA, under USDA-APHIS permit (P526P-19-02285). Within I&Q, the wood was placed inside multiple insect-rearing cages (BugDorm, Mega View Science Co., Ltd., Taichung, Taiwan) and maintained at room temperature for several weeks. Cages were checked daily for natural enemy emergence and any live parasitoids were collected using an aspirator. The majority of emerged parasitoids were used to initiate colonies on PSHB-infested wood material, though a small fraction of live parasitoids were collected directly into 2.0 mL screw cap tubes containing >95% ethanol, and stored at -20ºC for molecular identification. Dead parasitoids from rearing cages were later subjected to the same treatment and stored at -20ºC. Ethanol-preserved individuals were subsequently sorted into the following parataxonomic groups: 1) *Eucosmophorus* sp. and *Phymastichus* sp. with a proven parasitic association with the *Euwallacea fornicatus* species complex, i.e., those taxa emerging from experimental SHB-infested rearing logs (Chapter 1); 2) bethylid species with and without confirmed association; 3) other species from parasitoid families known to contain species associated with scolytine beetles; 4) parasitoids from families without any associated hosts in the family Curculionidae (e.g., Aphelinidae).

Along with the parasitoids collected from infested wood in I&Q, additional individuals of *Eucosmophorus* sp. were retrieved from sticky panel traps used in a study measuring seasonal flight dynamics of their beetle host (Liu et al., 2022). Throughout that study, *Eucosmophorus* sp. were retrieved each month from the traps using a drop of D-limonene to dissolve the surrounding glue. The specimens from each trap were subsequently preserved in 2.0 mL screw cap microcentrifuge tubes with 95% ethanol and

shipped to UCR in February 2022. On arrival at UCR, any residual glue was removed from the specimens. The ethanol was decanted from each vial and replaced with 1mL D-limonene. The vials were then placed in a tube rotator and rotated end over end for 10 minutes (@ 40 rpm). The D-limonene was decanted, replaced with 100% ethanol, and the specimens "washed" by rotating the vials for a further 5 minutes. This wash-step was repeated once more with 95% ethanol, after which, the ethanol was again replaced with fresh 95% ethanol. Vials were subsequently stored at -20ºC. This field-caught by-catch provided the opportunity to test the range of target DNA detection in field collected vs. lab-reared parasitoids.

Parasitoid DNA extraction

We initially utilized DNA extractions from our earlier study (Chapter 1). In that work, the DNA of each individual parasitoid was extracted using the HotSHOT non-destructive DNA extraction method (Truett et al., 2000). Those extractions were not performed specifically for use in this assay, and as such, precautions were not taken to prevent/remove surface contamination of the specimens with residual DNA from SHBs that may have been incidentally acquired (e.g., by coming into contact with SHB frass in or on the infested logs). Instead, parasitoids were simply removed from ethanol and allowed to air-dry on sterile filter paper for several minutes. Individual specimens were then placed into 0.5 mL microcentrifuge tubes containing 60 µL of a lysis buffer containing 25 mM NaOH and 0.2 mM disodium EDTA (pH 12), and incubated in a dry-bath for 30 min at 95ºC. Tubes were spun down and the reaction was neutralized with the addition of an equal volume of 40 mM Tris-HCl (pH 5) with a final volume of 120 uL.

The majority of *Phymastichus sp*. and bethylid wasps were extracted during this phase to determine ID and discern intraspecific genetic variation.

We were concerned that these initial specimens may provide false positives. Evidence of external DNA contamination has been previously reported on predators mass-collected via vacuum sampling or foliage beating (King et al., 2012; Greenstone et al., 2010). Therefore, we evaluated the potential for false positives in two ways. First, since our earlier collection contained lots of specimens of *Eucosmophorus* sp., many of those had not been extracted and remained in 95% ethanol. A few specimens of *Phymastichus sp*. were also left over from our earlier collections but unfortunately, all of the bethylids had already been extracted (see above). Thus, we took these residual wasps and surface-sterilized them using a dilute bleach solution following Greenstone et al. (2012). Parasitoids were placed in individual 1.5 mL microcentrifuge tubes containing 1 mL of a 2% bleach solution and slowly rotated (end over end) for 40 minutes in a tube rotator. Specimens were retrieved from the diluted bleach solution, rinsed on a Kimwipe® (Kimberly Clark Professional, Roswell, GA) using 95% ethanol, placed in a new 1.5 mL microcentrifuge tube containing 95% ethanol, and vortexed gently for 5-10 s. DNA was then immediately extracted from each parasitoid using the HotSHOT method as described above. The success of each DNA extraction was confirmed by amplifying the COI via PCR (see Chapter 1). Only surface-sterilized extractions that yielded a visible band on an agarose gel were tested using the qPCR assay.

The second approach we used to evaluate the potential for the production of false positives in our original set of specimens involved extracting DNA from PSHB frass

alone, and from a surrogate parasitoid (see above) deliberately contaminated with frass recently expelled from active PSHB galleries. For frass alone, individual "noodles" extruding from active PSHB galleries (n=2) were collected directly into microcentrifuge tubes containing 150 μL of HotSHOT lysis buffer, and immediately subject to extraction as described above, resulting in overall extraction volumes of 300 μL. To obtain "contaminated" parasitoids, two 15 mL centrifuge tubes were each populated with 24 adult *T. radiata* (from a UCR colony) and 5 grams of fresh PSHB frass (collected from colony cages), for 24 hours. The parasitoids were then euthanized in 95% ethanol and immediately subject to individual HotSHOT DNA extraction (as described above). Parasitoids from the first tube were extracted without further handling (i.e. replicating our initial extractions), but those from the second tube were surface-sterilized with 2% bleach (as described above).

Detection of shothole borer DNA within parasitoids

The qPCR conditions and Rotor-Gene settings used throughout the remainder of the study were as described above. The DNA extracted from each parasitoid specimen was initially tested in three replicate reactions. Specimens yielding a positive detection in all three replicates were scored as positive for SHB DNA, while those that were negative in all three replicates were immediately scored as negative for SHB DNA. If specimens yielded contradictory results across the three replicates, they were tested a second time, this time with two further replicate reactions. Each 72-reaction assay run contained duplicate standards of known beetle DNA concentration (wells 1-12) and non-template controls (wells 69-72). Similar to Goldman (2017), software was set to remove 2% of

outliers followed by the selection of "Noise Slope Correction" to remove noticeable slopes during initial cycles. These settings improve the visualization of raw data by minimizing the background noise prior to the amplification of the first Ct value in the standard controls.

Statistical analysis

Cycle threshold and slope values generated from Qiagen Rotor-Gene Q software were collected from all 12 runs and analyzed using R (version 4.1.2, R Core Team, 2021) and GraphPad Prism (version 9.0). A fixed effect (type III) one-way ANOVA, with the Geisser-Greenhouse correction was performed followed by Tukey's multiple comparison test to determine the level of variance across and within each standard dilution series.

Results

Optimization of fluorescent probe-based assay

We designed a fluorescent probe-based assay with the goal of detecting host DNA from the *E. fornicatus* cryptic species complex within a select species of parasitoids that emerged from native infested wood material. For this assay, we tested three different primer sets and selected the set that produced the best host DNA amplification. The assay was run 12 times along with the appropriate standards of diluted beetle DNA within *T. radiata* DNA. As expected, in each run, Ct values increased as the concentration of PSHB DNA decreased (**Figure 2.2**); fixed effect (type III) one-way ANOVA, with the Geisser-Greenhouse correction, average slope value of standards across 12 runs was 3.588 with a significant linear trend ($p < 0.0001$). Tukey's multiple comparisons test

showed significant differences between the Ct values of each dilution standard ($p = 0.0001$; **Figure 2.3**). Across 12 different runs, reaction efficiencies typically ranged between 90 and 100%, and across all runs, efficiency averaged 96% (**Figure 2.3**). Although one "outlier" run produced an efficiency of 85%, and another of 135%, this did not result in any significant shift in the Ct values produced by each standard dilution across the 12 runs ($p = 0.9031$; **Figure 2.3**). Average Ct values for undiluted PSHB DNA and the dilution standards are given in **Table 2.1**. The limit of detection for PSHB DNA within that of *T. radiata* DNA was determined to be 10^{-5} PSHB dilution, which will generate an average Ct value of 36.95 (± 1.80). The 10^{-6} dilution produced sporadic Ct values in the range 37-40, but the majority of replicates resulted in zero detectable amplification. Thus, reactions that produced Ct values of less than or equal to 37 (the lower the Ct value, the higher the host DNA concentration) were considered to be positive for host DNA (i.e. *E. fornicatus* species complex DNA), and those higher than 37 were considered negative.

Detection of host DNA within associated parasitoids

Of the 200 non-surface sterilized specimens tested in total, 80 were positive in all three initial replicates, and 70 were negative in all three initial replicates. The remaining specimens were positive in only 1 or 2 of the initial three replicates, typically with a Ct value close to 37, and were subjected to a further two replicate assays. Those specimens that tested positive at least once in those further replicates were designated as positive for SHB detection. The following parasitoids amplified host DNA: *Eucosmophorus sp.* (GenBank COI and 28S accessions OP933742 and OP924525, respectively),

Phymastichus sp.(GenBank COI and 28S accessions OP933749 and OP924535, respectively), *Plastanoxus sp*. (GenBank COI and 28S accessions OP933738 and OP924521, respectively) and a *Cosmophorus* nr. *sp*. (GenBank COI and 28S accessions OP933741 and OP924524, respectively). None of the other bethylid species (GenBank accessions COI: OP933734-OP933737, 28S: OP923517-OP924521, OP924523) resulted in a positive detection of SHB DNA. Furthermore, parasitoids that emerged from native wood material presumed to not have any direct association with the *E. fornicatus* species complex (i.e. aphid and scale insect parasitoids; **Table 1.2**) did not amplify any SHB DNA.

The Ct values of specimens that tested positive for host DNA typically ranged between 30 and 37, with the exception of one male *Eucosmophorus* specimen that had a Ct value of 22.18 (**Table 2.2**). The specimen was retested 8 times, with each replicate consistently matching the previous result of high amounts of host DNA. Of the *Eucosmophorus* specimens tested after the bleach treatment, 10 of 27 specimens amplified host DNA making 63 (from 139) specimens that amplified, in total. The average Ct value of *Eucosmophorus* specimens that were surface sterilized with bleach (collected from white panel traps) was 34.34 with 30.72 being the lowest Ct value. The earlier samples that were not bleached had an average Ct value of 33.36 with the lowest Ct value being 30.55, excluding the previously mentioned male specimen. Similarly, of the three "leftover" *Phymastichus* wasps, two amplified host DNA in the Ct range observed previously within this genus.

Potential for diagnostic assay to produce false positives

Both DNA extractions of frass freshly extruded from PSHB galleries produced a positive result using the assay. However, in the companion experiment where we deliberately tried to contaminate *T. radiata* individuals with PSHB frass, only 4 of 24 specimens that did not undergo surface sterilization (bleach treatment) produced a positive result, with Ct values ranging from 33.63 to 36.98. None of the 24 specimens that did undergo the bleach treatment, showed any evidence of PSHB DNA. Upon close inspection, the HotSHOT DNA extractions of the specimens that did produce detectable host DNA retained visible fragments of frass still adhered to the wasp specimen.

Discussion

Parasitoid wasps are the most common focus of biological control research (Greathead, 1986), yet the host-parasitoid interactions of many potential biological control agents are poorly understood. Traditional mapping of host-parasitoid interactions is confirmed either by visual observation, or through multiple rearing attempts to establish a parasitoid colony. These conventional methods become more challenging when dealing with species with a hidden life-style such as the ones in the *E. fornicatus* cryptic species complex. The short amount of time the beetles are detected outside their galleries creates only a small window in which to accurately identify associated natural enemies based purely on observation. As an alternative, we developed a molecular assay to investigate whether or not DNA from the beetles could potentially be detected in the gut of adult parasitoids, thereby revealing a trophic link between parasitoid and host. This

was based on amplification/detection of a short fragment of beetle DNA in DNA extractions of parasitoids that emerged from wood material infested with beetles in the *E. fornicatus* species complex.

Successful amplification of PSHB DNA from DNA extractions of three parasitoid wasps identified in Chapter 1, corroborated their role as candidates for the biological control of beetles in the *E. fornicatus* cryptic species complex. This also further confirmed that the molecular gut content assay we designed could be used for establishing cryptic links between adult parasitoids and their hosts. However, the assay was not 100% reliable. We were confident that every *Eucosmophorus* adult wasp, that we reared from PSHB infested logs in the lab, had parasitized and consumed SHB DNA at some point, despite host DNA being detected in 45% of the samples. The negative samples may be the results of the voiding host DNA from the digestive system of the wasps. It is to be expected that if a wasp is no longer in contact with the host the DNA concentration will decline to undetectable levels. To our knowledge, this study is one of the few that investigated the detection rates of consumed host DNA within parasitoid wasps post eclosion (also see, Rougerie et al., 2011)

Since the majority of earlier molecular gut content analyses focused on predator-prey interactions (rather than parasitoid-host interactions), our initial focus of this study was to test the bethylid species that emerged from shothole borer-infested wood material for evidence of host consumption. The interest in bethylids stems from their behavior as both aggressive predators on immature stages of the host and as parasitoids on older, more suitable stages of the host (Clausen, 1940). However, of the individuals tested,

Plastanoxus sp. was the only bethylid wasp that generated a 60% host detection rate. The low positive detection rates in the additional bethylid species was likely due to either 1) low sample size to test, 2) the false-negative effect previously mentioned, or 3) simply, not being a parasitoid associated with shothole borers. Additional specimens will be required to reach a sound conclusion. Similar to the pitfalls behind the low mass-rearing success of *Plastanoxus* parasitoids (**Chapter 1**), the low, or absence of, amplified host DNA may be due in part to wasps not host feeding due to depleted food source (i.e. beetle eggs and early larval instars) near the end of the parasitoids' lifespan. While *Phymastichus* wasps had a shorter lifespan than bethylids, 62.5% of tested specimens detected target host DNA yet in a similar range of Ct values seen with *Plastanoxus* wasps.

The majority of parasitoids tested in the molecular assay were *Eucosmophorus* sp. simply because that was the most abundant species that emerged from PSHB-infested logs (see Chapter 1), and the only species retrieved from sticky traps. Among the *Eucosmophorus* specimens, those that were surface sterilized with bleach yielded similar Ct values to those without a bleach treatment, suggesting that surface sterilization was unlikely to affect the outcome of individual assays. However, in *T. radiata* that were intentionally exposed to beetle frass over a 24-hour period, 4 from 24 (16%) of non-surface sterilized specimens amplified target host DNA suggesting that false positives were possible as a result of contamination with PSHB frass. Thus, we recommend that specimens should be surface sterilized prior to future assay runs. One male specimen surprisingly yielded a Ct value of 22.18, indicating the presence of a substantial amount

of PSHB DNA. We assayed this specimen in a total of 8 replicates, each one producing a similar result. We believe that the low Ct value produced by this specific specimen could be indicative of a very recent eclosion from its host carcass that was immediately preserved in ethanol as one of the few live samples selected for molecular identification. Another possibility is that the adult male *Eucosmophorus* had very recently host-fed on a shothole borer adult, larva, or egg. While host-feeding is not uncommon with parasitoids, little is known about the phenomenon in this *Eucosmophorus* sp. Further investigation with additional samples is required to test this theory.

While our non-destructive DNA extraction protocol from all tested parasitoids yielded an ample concentration and quality for successful PCR and sequencing of the wasp DNA, DNA from most molecular gut content analyses in both predators and parasitoids is extracted destructively using either partial (usually abdomen) or whole specimens (King et al., 2008; Rougerie et al., 2011). Whether a destructive DNA extraction, in which the parasitoid is ground up, would produce different results is unknown. Perhaps grinding a specimen would expose more tissues to the extraction buffers allowing for greater recovery of DNA from the gut. That said, in a recent study that utilized both destructive and non-destructive DNA extraction methods, Batovska et al. (2021) were still able to detect the desired rare target species in their metabarcode analysis of DNA acquired through the non-destructive approach. In practice, further testing is recommended to compare Ct values of target host DNA within that of their parasitoid DNA in both destructive and non-destructive samples across all parasitoids associated with *E. fornicatus* cryptic species complex.

References

Abraham, Y., Moore, D., & Godwin, G. (1990). Rearing and aspects of biology of *Cephalonomia stephanoderis* and *Prorops nasuta* (Hymenoptera: Bethylidae) parasitoids of the coffee berry borer, *Hypothenemus hampei* (Coleoptera: Scolytidae). *Bulletin of Entomological Research*, *80*(2).

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990) Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403-410.

Beaver, R.A. (1988), Biological studies on ambrosia beetles of the Seychelles (Col., Scolytidae and Platypodidae). *Journal of Applied Entomology*, *105*, 62-73.

Browne, F.G. (1961), The biology of Malayan Scolytidae and Platypodidae. *Malayan Forest Records*, 22, 1– 255.

Clausen, C. P. (1940). *Entomophagous insects*. McGraw-Hill book Company, Incorporated.

Eatough Jones, M., & 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.

Escobar-Ramírez, S., Grass, I., Armbrecht, I., & Tscharntke, T. (2019). Biological control of the coffee berry borer: main natural enemies, control success, and landscape influence. *Biological control*, *136*, 103992.

Goldman, A. (2017). *Predators of Asian Citrus Psyllid (Diaphorina citri) in Southern California*. [Unpublished dissertation, University of California, Riverside]. ProQuest Dissertations and Theses database.

Greathead, D. J. (1986). Parasitoids in classical biological control. In *Insect parasitoids. 13th Symposium of the Royal Entomological Society of London, 18-19 September 1985 at the Department of Physics Lecture Theatre, Imperial College, London* (pp. 289-318). Academic Press.

Greenstone, M. H., Szendrei, Z., Payton, M. E., Rowley, D. L., Coudron, T. C., & Weber, D. C. (2010). Choosing natural enemies for conservation biological control: use of the prey detectability half-life to rank key predators of Colorado potato beetle. *Entomologia Experimentalis et Applicata*, *136*(1), 97-107.

Greenstone, M. H., Weber, D. C., Coudron, T. A., Payton, M. E., & Hu, J. S. (2012). Removing external DNA contamination from arthropod predators destined for molecular gut-content analysis. *Molecular Ecology Resources*, *12*(3), 464-469.

Hall, T.A. (1999). BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. Nucleic Acids Symposium Series, *41*, 95-98.

King, R. A., Read, D. S., Traugott, M., & Symondson, W. O. C. (2008). INVITED REVIEW: Molecular analysis of predation: a review of best practice for DNA-based approaches. *Molecular ecology*, *17*(4), 947-963.

King, R. A., Davey, J. S., Bell, J. R., Read, D. S., Bohan, D. A., & Symondson, W. O. C. (2012). Suction sampling as a significant source of error in molecular analysis of predator diets. *Bulletin of Entomological Research*, *102*(3), 261-266.

Liu, F. L., Rugman-Jones, P., Liao, Y. C., Husein, D., Liang, H. H., Tuan, S. J., & Stouthamer, R. (2022). Seasonal Dynamics of Flight Phenology of the Euwallacea fornicatus Species Complex and an Associated Parasitoid Wasp in Avocado Groves in Taiwan. *Journal of Economic Entomology*.

Mutanen, M., Ovaskainen, O., Várkonyi, G., Itämies, J., Prosser, S. W., Hebert, P. D., & Hanski, I. (2020). Dynamics of a host–parasitoid interaction clarified by modelling and DNA sequencing. *Ecology letters*, *23*(5), 851-859.

Peña, J. E., Weihman, S. W., McLean, S., Cave, R. D., Carrillo, D., Duncan, R. E., Evans, G., Krauth, S., Thomas, M. C., Lu, S. S., Kendra, P. E., & Roda, A. L. (2015). Predators and parasitoids associated with Scolytinae in *Persea* species (Laurales: Lauraceae) and other Lauraceae in Florida and Taiwan. *Florida Entomologist*, *98*(3), 903-910.

Rougerie, R., Smith, M.A., Fernández-Triana, J.L., Lopez-Vaamonde, C., Ratnasingham, S., & Hebert, P.D. (2011). Molecular analysis of parasitoid linkages (MAPL): gut contents of adult parasitoid wasps reveal larval host. *Molecular Ecology, 20*.

Stouthamer, R., Rugman-Jones, P., Thu, P. Q., Eskalen, A., Thibault, T., Hulcr, J., Wang, L., Jordal, B. H., Chen, C., Cooperband, M., Ling, C., Kamata, N., Lu, S., Masuya, H., Mendel, Z., Rabaglia, R., Sanguansub, S., HsinHui, S., Sittichaya, W., & Zong, S. (2017). Tracing the origin of a cryptic invader: phylogeography of the *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae) species complex. *Agricultural and Forest Entomology*, *19*(4), 366-375.

Truett, G. E., Heeger, P., Mynatt, R. L., Truett, A. A., Walker, J. A., & Warman, M. L. (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques*, *29*(1), 52-54.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M and Rozen SG. 2012. Primer3--new capabilities and interfaces. Nucleic Acids Res. 40:e115.

Wirta, H. K., Hebert, P. D., Kaartinen, R., Prosser, S. W., Várkonyi, G., & Roslin, T. (2014). Complementary molecular information changes our perception of food web structure. *Proceedings of the National Academy of Sciences*, *111*(5), 1885-1890.

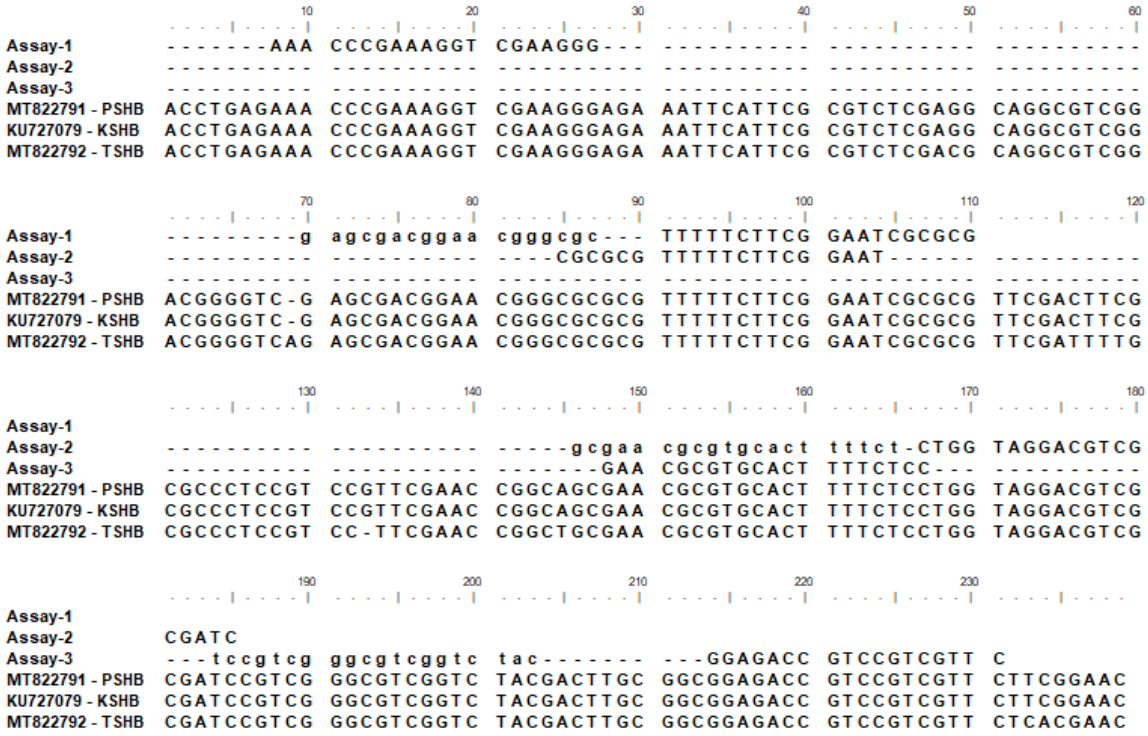

Figure 2.1 Three potential TaqMan assays for the universal detection of 28S-D2 DNA of different Shot-hole borer species. DNA sequences of the different shot-hole borer species are also shown with their GenBank accession numbers. Primers are shown in uppercase, probes in lowercase. Assay-3 was developed further.

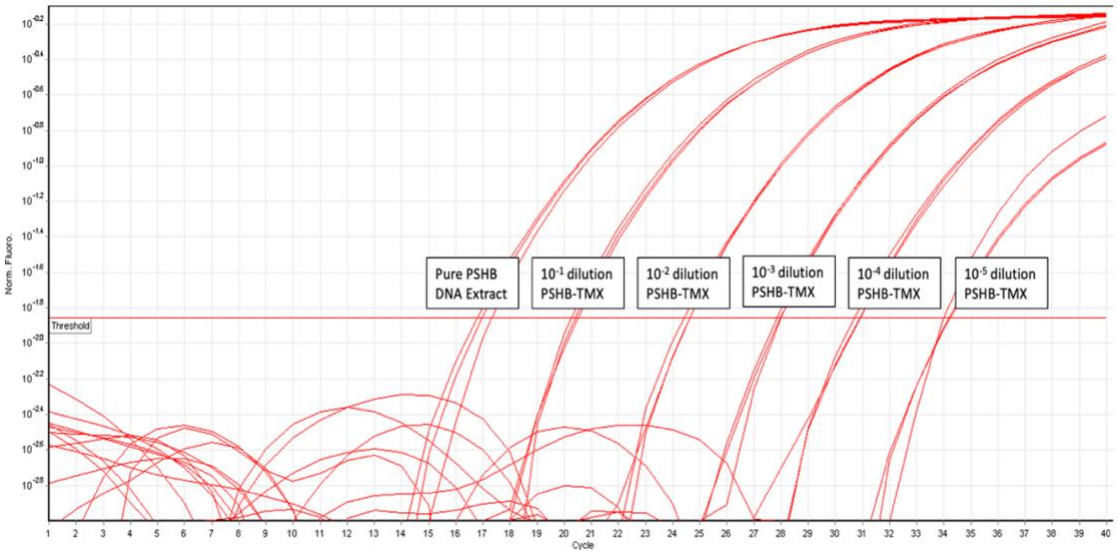

Figure 2.2. qPCR TaqMan assay displaying amplification curves of PSHB 10-fold diluted series within *Tamarixia radiata*. Low cycle threshold (Ct) values are invertedly reciprocal to high concentration of target (PSHB) DNA.

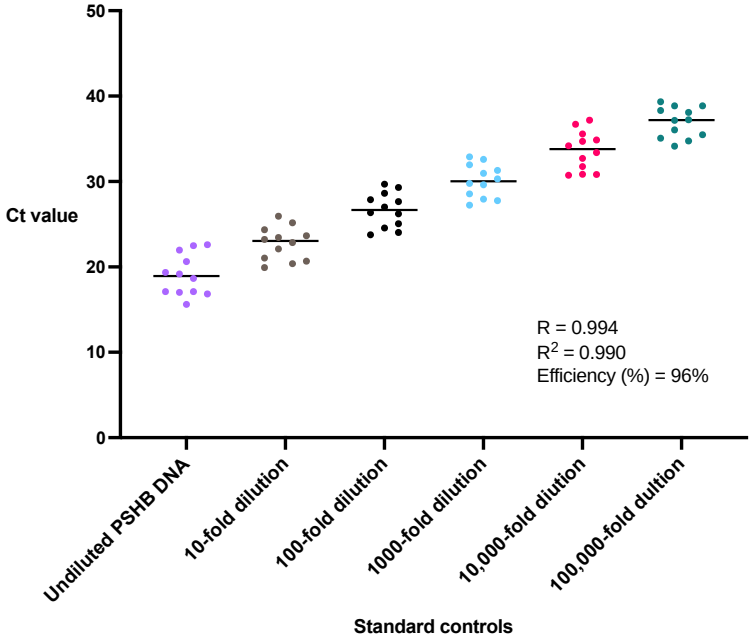

Figure 2.3. Variation within and between the Ct values resulting from a standard dilution series of PSHB DNA in that of a surrogate parasitoid, *Tamarixia radiata*.

Table 2.1. Evaluated assay using standards consisting of undiluted DNA of PSHB and a tenfold gradient dilution of PSHB DNA in that of *Tamarixia radiata* DNA. Dilution series ranged from 10^{-1} to 10^{-5}. Ct values are presented as the mean ± *SD* (*N* = 12).

Standard Controls	**Ct**
Undiluted PSHB DNA	19.05 ± 2.41
10^{-1} PSHB dilution	22.73 ± 1.94
10^{-2} PSHB dilution	26.67 ± 2.03
10^{-3} PSHB dilution	30.07 ± 1.93
10^{-4} PSHB dilution	33.62 ± 2.28
10^{-5}PSHB dilution	36.95 ± 1.80
10^{-6} PSHB dilution	—*

*Trace DNA of PSHB was not detected or simply did not amplify.

Table 2.2. qPCR TaqMan assay using species specific primers to detect shothole borer DNA within parasitoid DNA that 1) was extracted prior to bleach treatment, or 2) were caught in quercivorol baited yellow panel traps. Low cycle threshold (Ct) inversely represents high fluorescence detection of SHB DNA.

Parasitoid species	Total specimens tested	Specimens with traces of SHB DNA	Ct range value	Ct average value	Ct value with highest target DNA detected
Eucosmophorus sp.	139	63 (45.2%)	1K-10K 10K-100K ~100K	34.89 ± 3.11	22.18*
Phymastichus sp.	16	10 (62.5%)	10K-100K ~100k	34.58 ± 1.36	33.19
Plastanoxus sp.	17	9 (52.9%)	10K-100K ~100k	35.35 ± 1.39	32.91
Cosmophorus nr. *sp.*	2	2	10K-100K ~100K	35.35 ± 2.28	31.98

* Specimen was tested 8 times

Chapter 3: Hitchhiker's guide to the gallery: A closer look at nematodes associated with the *Euwallacea fornicatus* Species Complex and their fitness costs under laboratory settings.

Abstract

Ambrosia beetles consist of roughly 3500 described species worldwide with the majority typically residing in dying or dead host plants, and being of little economic importance. In contrast, the polyphagous shothole borer (PSHB), *Euwallacea fornicatus*, is an invasive ambrosia beetle responsible for causing damage to urban trees and economic crops in southern California, Israel, South Africa and, more recently, Australia. This species is just one of a group of cryptic species belonging to the *Euwallacea fornicatus* species complex. The biology and lifestyle of PSHB make it difficult to control by chemical means and, as such, biological control is seen as a valuable management option. Thus, foreign exploration for natural enemies of PSHB in Taiwan, part of the beetles' native range, was initiated. While the focus of exploration efforts has been on parasitoid wasps, entomopathogenic nematodes have been commonly encountered, raising questions about their potential role in the rise and decline of beetle populations in the environment. In this study, we record a series of nematode species, in the families Aallantonematidae, Aphelenchoididae, and Diplogastridae, that appear to have a phoretic relationship with PSHB. We subsequently test the fitness costs associated with one nematode species, found in both Taiwan and California, using PSHB colonies reared in semi-artificial diet. Our results show that the overall count of PSHB broods and

the number of those that survived was significantly reduced in colonies harboring nematodes, and larval count was negatively impacted by nematodes, with a two-fold reduction.

Introduction

Ambrosia beetles in the subfamily Scolytinae comprise of roughly 3500 described species (Wood & Bright, 1992). While the majority of ambrosia beetles are of little economic importance as they inhabit the xylem tissues of dying or dead host plants, a few have become established pests of forest and commercial crops (Hulcr et al., 2017). The polyphagous shothole borer (PSHB), *Euwallacea fornicatus* Eichhoff (Coleoptera: Scolytinae), is an invasive ambrosia beetle that has gained attention in southern California after signs of damage were observed in urban trees and the important economic crop avocado (Eskalen et al., 2013). Outbreaks in Israel and South Africa have reported similar damage on residential trees and avocado groves (Mendel et al., 2012, Paap et al., 2018). *Euwallacea fornicatus* is one of four species currently recognized in the *Euwallacea fornicatus* cryptic species complex, which collectively are commonly referred to as shothole borers (SHBs). Members of this species complex are known to have an obligate association with a *Fusarium* "ambrosia" fungus that they utilize as the main food source for the brood larvae and adults (Batra, 1966). Spores of the symbiotic fungi are carried in internal vestigial pockets (mycangia), and are inoculated into the xylem tissue of the host plant as the beetles excavate their galleries. *Fusarium* fungi are pathogenic to some host plant species, and, together with the accrued physical damage from high beetle infestation levels, can induce Fusarium Dieback, which can lead to tree mortality (Eskalen et al., 2012).

Control methods for SHBs are limited due to the beetles' cryptic life style that predominantly takes place inside the host plant. Upon emergence from their natal gallery, adult females, which have already mated with a male sibling, typically excavate a new gallery either on the same host plant, or one nearby. Their short exposure outside the gallery limits the implementation of chemical control. Furthermore, although not entirely without merit (Eatough Jones and Paine, 2018), chemical control methods are costly, and largely impractical due to human and environmental concerns over accumulated pesticide residue and chemical runoff. This has led to interest in using classical biological control to combat SHBs, and a recent study has identified several species of parasitoid wasp that attack SHBs in their native range (Husein et al., in review). That study involved importing SHB-infested wood from Taiwan into the U.S., and collecting potential natural enemies as they emerged from that wood. While the study focused on the identification of arthropods, we also recovered several nematode species. Interestingly, nematodes are particularly common associates of scolytid beetles and it has been hypothesized that entomopathogenic nematodes (ones that live on or inside the bodies of insects) can influence the rise and decline of beetle populations (Massey, 1974).

There are an estimated 40,000-500,000 species of insect-associated nematodes worldwide (Giblin-Davis et al., 2013), with scolytine beetles being one of the most important and suitable hosts, since their habitat provides optimum environmental conditions for nematodes to thrive and persist (Browne, 1961). Interest in entomopathogenic nematodes, with a biological control view, did not gain momentum until the 1950s (Ruhm, 1956). Subsequently, several extensive surveys have been

conducted documenting nematodes that are commonly found in association with scolytine bark beetles in Europe and North America (Ruhm 1956; Massey 1974; Poinar 1975). These have focused on the beetle genera *Ips* (Ruhm, 1954, 1955, 1956; Massey, 1960; Hoffard and Coster, 1976), *Dendroctonus* (Massey, 1956, 1966; Furniss 1967; Thong and Webster, 1983), *Pityogenes* (Reid, 1958; Nickle, 1963), and *Scolytus* (Massey 1964; Ashraf and Berryman, 1970; Hunt and Hague, 1974; Moser et al., 2005). In contrast, very few surveys have been done in relation to scolytine ambrosia beetles (Weber & McPherson, 1983; Castillo et al., 2002; Kanzaki et al., 2011; 2014; 2013;). Furthermore, much of the biological control research effort related to nematodes associated with scolytine beetles has focused on species in the families Heterorhabditidae and Steinernematidae, due to their wide host range (Nermut et al., 2012), or on more discernible, endoparasitic nematodes such as *Contortylenchus* and *Parasitylenchus spp*. (Weiser et al., 2006; Takov et al., 2006). This has limited the identification and diagnosis of the remaining nematode groups.

It has been suggested that there may be as many as 2 to 3 unique (species-specific) nematodes associated with each species of ambrosia beetle. Documented relationships of nematodes with bark and ambrosia beetles have varied from parasitism, mutualism, and, most commonly, commensalism in the form of phoresy (Ruhm, 1956; Massey, 1974; Poinar, 1975; Kaya and Stock, 1997; Giblin-Davis et al., 2013). Phoretic nematodes are carried by their beetle hosts, either beneath the elytra in specialized pockets called nematangia, in intersegmental folds of the abdomen and legs, or on the surface of the exoskeleton (Poinar, 1975; Cardoza et al., 2006). In addition, these

nematodes have been reported to be found internally, within their host's trachea, head glands, and malpighian tubules (Massey, 1974; Poinar, 1975). Yet, despite their frequent occurrence, whether on or within their hosts, phoretic nematodes are typically regarded as harmless to the beetle, since they are dependent on the fitness and successful dispersal of their hosts, in order to reach new habitats (Signe White et al., 2017). This assumption can be misleading for symbionts in general. The costs/benefits of these relationships can change, and levels of virulence may evolve, in response to new environmental factors, which can potentially occur with phoretic nematodes, as a response to resource scarcity in the habitat of their host (Alizon, 2008; Little et al., 2008). Relatively few studies have determined the fitness cost of phoretic nematodes on their hosts, and consequently the assumption of neutrality in this relationship is unproven. Further investigations are needed.

In 2016, nematodes were recovered from several lab colonies of PSHB initiated with beetles collected from an invasive population in southern California. We originally hypothesized that the association was purely phoretic, since neither mortality, nor immediate reproductive effects, were observed when the nematodes were introduced to individual beetles on potato dextrose agar (PDA) plates inoculated with *Fusarium euwallacea* Freeman, Mendel, Aoki, and O'Donnell, the primary symbiotic fungus of PSHB (Husein, personal observation). However, the same nematode species was recovered in 2021 from a separate PSHB colony line, also originally collected from southern California. However, the beetles were from a region where the numbers of PSHB caught in monitoring traps was in sharp decline. Furthermore, during foreign

exploration for arthropod natural enemies of SHBs in 2017, multiple species of nematodes, including the same species that we found in southern California, were recovered from adult beetles that emerged from imported wood material collected from their native range in Taiwan. Herein, we document a series of nematodes associated with the *E. fornicatus* species complex, and compare our findings to previous nematode surveys conducted in Taiwan. In addition, we conduct a series of laboratory assays to assess the fitness costs incurred by PSHB as a result of association with the one nematode species that we know is already in California. The objectives were: 1) to identify nematodes associated with the *E. fornicatus* species complex, and 2) to determine host development and fecundity effects of a California-established nematode species on PSHB colonies under laboratory settings.

Materials and Methods

Collection of SHB adults and initiation of experimental colonies

Between 2017 and 2019, logs infested with the *E. fornicatus* species complex were collected from two avocado orchards in Danei, Taiwan (**Table 3.1**), and imported to the Insectary and Quarantine (I&Q) facility located at the University of California, Riverside (UCR), USA, under USDA-APHIS permit P526P-19-02285. Imported wood species included avocado (*Persea* spp.), castor bean (*Ricinus communis* Linn), Chinese chestnut (*Castanea mollissima* Blume), lychee (*Litchi chinensis* Sonn.), and tea (*Camelia sinensis* L. Kuntze). Logs were placed individually into modified plastic containers as described in Husein et al (in review). Containers were checked daily and emerging adult

beetles were collected and either, immediately preserved in 95% ethanol for identification, or set aside to be reared in laboratory cultures. To mitigate transfer of contaminants and debris, lab colonies were initiated using mature adult females (fully sclerotized) that first were rinsed in 95% ethanol for 3-4 seconds, and then placed onto a 100 x 60 mm Petri plate with a folded Kimwipe (KIMTECH® Science, Kimberly-Clark Professional, Roswell, GA, USA), and briefly rinsed with ultrapure water. Females that survived the rinse process were introduced to individual semi-artificial sawdust diet tubes (Carrillo and Dodge et al., 2020), each representing an experimental colony, and maintained in I&Q insectary rooms (27 ± 2°C, 8L:16D). Lab colonies were similarly initiated using PSHB from locally (southern California) collected castor bean.

Nematode isolation, imaging, and the initiation of nematode stocks/colonies

Shothole borer colonies were examined weekly. The diet media, and occasionally beetle galleries, can be seen through the clear walls of the colony tubes. Colony tubes were observed under a stereo microscope and rotated by hand to enable viewing. In some of the experimental colonies, nematodes (in variable densities) became visible 3 to 4 weeks after the PSHB foundress was introduced to the diet (**Figure 3.1**). Using a disposable plastic transfer pipette, ~15 mL of ultrapure water was added to those tubes. The tubes were inverted several times, and nematodes, now suspended in the water, were decanted into a new 50 mL centrifuge tube. Since this initial suspension not only contained nematodes, but also beetle and diet debris, the liquid suspensions were subsequently poured into a 100 x 60 mm glass petri plate and the inside of the tubes was rinsed off with additional ultrapure water. The petri plates were momentarily swirled, and

then allowed to stand for 20 seconds, to allow the debris to settle. Using a new disposable transfer pipette, the water (still containing the suspended nematodes) was transferred to a fresh 15 mL tube, leaving behind as much of the unwanted residue as possible. The suspension then was aliquoted into 1.5 mL microcentrifuge tubes which were capped and centrifuged at 2500 rpm for 5 min to pellet the nematodes. The upper ~90% of the water from each tube was drawn off and discarded and replaced with an equal amount of clean water. This process was repeated until the suspension was clear, with no visible remains from the beetle diet. Aliquots of the "washed" nematodes (suspended in water) were subsequently stored at room temperature awaiting the next step.

Petri plates containing nematode growth medium (Teknova Inc, Hollister, CA, USA) were prepared as per the manufacturer's instructions, with the final concentrations: 0.25% Tryptone, 0.30% NaCl, 2.00% Agar, 0.11% K_2HPO_4, 0.25% KH_2PO_4, 5 µg/ml Cholesterol, 1 mM $MgSO_4$, 1 mM $CaCl_2$. The plates were inoculated with 0.5 µL of OP50 (a harmless strain of *E. coli* that provides additional food) and incubated at 37°C overnight. Ten microliters of each washed nematode suspension then was transferred to an individual growth plate, to initiate laboratory populations. Populations were subsequently maintained by transferring nematodes to fresh plates, seeded with *E. coli*, every 5-7 days.

The remaining nematodes in the pellets were resuspended and transferred back to a 15 mL tube with 5 mL of ultrapure water, and incubated in a 60°C water bath for 30-60 min. To properly heat fixate the nematodes, an equal amount of a 10% buffered formaldehyde solution (pH 7.0) was added to the nematode suspension. A representative

set of nematodes isolated from different regions, different wood species, and on different dates, were slide-mounted and photographed under a compound microscope at variable magnification starting at 60X. Additional images, mainly of the locally established nematode species, were captured with a Leica M10 with a Z16 APO A 271 microscope, and stacked using Zerene Stacker (ver. 104, Zerene Systems, LLC). Nematodes were subsequently sorted into a series of morphotypes for molecular identification.

DNA-based identification of nematodes and their SHB hosts

A sample of nematodes from each laboratory colony was collected and placed into a 1.5 mL microcentrifuge tube containing 100% ethanol. The nematodes were pelleted in a centrifuge and the ethanol was decanted. The tubes were then incubated, un-capped, at 56°C for 15 minutes, to allow any residual ethanol to evaporate. DNA was subsequently extracted from the dried nematode samples using the HotSHOT method (Truett et al., 2000). In addition, nematodes found directly on SHBs that emerged from native infested wood material, or from the colony tubes, were collected by rinsing the host beetle in 95% ethanol. The DNA of these nematodes was extracted as described above, but the DNA of the host SHB was also extracted in a separate HotSHOT reaction. The 28s-rDNA nuclear region was amplified using primer pairs 28sF3633 and 28sR4076 (Rugman-Jones et al. 2010). The mitochondrial gene cytochrome oxidase c subunit 1 (COI) was amplified using primer pairs LCO1490 and HCO2198 (Folmer et al. 1994). Gene amplification of 28s and COI were done following protocols described in Stouthamer et al. (2017). The 18s rRNA nuclear gene was amplified using primer pairs 18S-F and 18S-R (Liu et al., 1997) following the same protocol used for 28s-rDNA.

Reactions were done in a volume of 25 μL, which included 2 μL of template DNA. PCR products were checked using a 1% agarose gel stained with ethidium bromide. Successful amplicons were cleaned with the Zymo DNA Clean & Concentration- 5 kit (Zymo Research Corporation, Irvine, CA, USA), and sent to the Institute of Integrative Genome Biology at UCR for sequencing. Forward and reverse reads were checked for parity using the software BioEdit 7.0.5.3 (Hall, 1999). Molecular identification of each nematode species was attempted using BLASTn searches of the NCBI GenBank database.

Fitness costs of nematode infections

While multiple nematode species were isolated from Taiwanese SHB, one species, *Rhabditolaimus* sp., was far more numerous than the others (see Results). Not only was that species common in Taiwan, it was also present on SHB from California, and was collected here as early as 2016. We were interested in investigating the role that this nematode might play in regulating population densities of invasive shothole borers. To investigate fitness costs that might be associated with this nematode (**Nematode species 1, Table 3.2**), beetles from California lab-reared PSHB colonies, without signs of nematode infestation, were used to initiate a multitude of experimental PSHB colonies. A single mature female adult PSHB was added to a semi-artificial diet tube and randomly assigned to a treatment or control group. Meanwhile, nematode growth media plates with active nematode populations were flipped upside-down overnight with 3 mL of water aliquoted at the bottom of the petri plate. Naturally, the nematodes migrated to the water source and were collected in 1.5 mL capped tubes. To remove debris from the media plates, tubes were centrifuged for 30 seconds at 2500 rpm where the supernatant was

removed, replaced with PBS (phosphate-buffered saline), and vortexed for 10 seconds. This process was repeated three times with PBS and three more times with water. After the last vortex run, 100uL of the suspension were aliquoted onto a clean petri plate. The number of nematodes present were counted using a tally counter. This provided an estimate of the number of nematodes anticipated per 10 μl aliquot when introduced to beetle colony tubes. Once twenty-four hours have passed for female PSHB adults to initiate their colonies, the tubes in the treatment group were inoculated with ~500 nematodes suspended in a 10 μl drop of water. Control group tubes received a 10 μl drop of nematode-free water. Treatment and control groups each contained 25-50 tubes and the entire experiment was replicated 10 times (trials). Colonies were maintained at 27 ± 2°C, 8L:16D, for the duration of the experiment. For each trial, approximately half (12-25) of the colony tubes from each group were dissected (see below) after 5 weeks, to give a tentative representation of the effect of nematodes on the success of the first offspring generation. Count data and analysis from this time point will be referred to "5-week." The rest were dissected after 8 weeks, at which point the colony is expected to contain a mix of first and second generation together. Count data and analysis of colonies dissected at the first 8-week time point will be referred to "8-week (continuous)." To investigate transgenerational fitness effects of nematodes on first generation beetles after their dispersal from their natal gallery, a number of mature adult female beetles, from a subset of the tubes dissected at 5 weeks, were transferred into new individual colony tubes. These female beetles were selected only from 5-week colonies that also contained at least one mature brother, with which it was assumed they had already mated. These females

were not sterilized with ethanol nor inoculated with more nematodes. The colonies from this setup were maintained for 8 weeks and later dissected as described above. The data collected from this group will be referred to as 8-week "dispersed."

Dissection involved removal of the diet from the tube, followed by its careful deconstruction, with the aid of forceps, under a stereo-microscope. All PSHB life stages (eggs, larvae, pupae, teneral adults, and mature adults) were counted, and all life stages except eggs were recorded as dead or alive. The obvious presence of nematodes in each colony also was recorded.

Statistical analysis

To determine the effect nematode presence had on the successful establishment of a PSHB colony, contingency of Chi squared analysis was performed by comparing the number of failed and successful PSHB colonies in both the control and nematode treatment group. Negative binomial regression was performed in Stata (16.1, StataCorp LLC, College Station, TX) to analyze the effects of nematodes on 1) the total PSHB count (viable and dead), 2) the viable PSHB count of all life stages, and 3) juvenile (larvae and eggs) count within established PSHB colonies. The appropriate count was the dependent variable, and treatment (nematode vs control), dissection time points (5-week, 8-week "continuous", and 8-week "dispersed"), and replicate (1-10) were fixed effects. Within the nematode treatment group, a two-way Analysis of Variance (ANOVA,Stata) was also used to compare the numbers of living offspring between colonies which at the time of dissection were obviously still infested with nematodes, and those that were not. Living offspring was the dependent variable, and dissection time point and

presence/absence of nematodes were included as fixed effects. Figures were produced using GraphPad Prism version 9.4.0 (GraphPad Software Inc., San Diego, California, USA, www.graphpad.com).

Results

Molecular identification of nematode species

Based on (a sometimes incomplete combination of) DNA sequences of two nuclear genes (18S [n=3] and 28S [n=5]) and one mitochondrial gene (COI [n=11]), the nematodes collected in this study harbored 12 different genetic signatures (taxa), each of which likely represents a different species (**Table 3.2**). These nematodes were variously isolated from PSHB and TSHB, but not KSHB (although the latter was rarely encountered; **Table 3.2**). Of the 12 genetic signatures, Blastn searches were able to unambiguously identify only one to species (*Bursaphelenchus penai* Kanzaki, Giblin Davis, Carillo, Duncan and Gonzalez). Three samples were determined to genus (*Rhabditolaimus* sp.1, *Aphelenchoides* sp.1, and *Bursaphelenchus* sp.1), one to family (Allantonematidae) and the remainder as belonging to the order Rhabditida (**Table 3.2**). This lack of resolution was due in part to a general paucity of nematode sequences in GenBank, particularly for COI, and the often conserved nature of 18S and 28S which resulted in identical levels of similarity to accessions from more than one species. Interestingly, *Rhabditolaimus* sp.1 was isolated not only from the Taiwan beetles, but also from PSHB field-collected in southern California (**Table 3.2**). The nature of the relationship between each nematode and its respective host is given in **Table 3.2,** and was

based either on visual observation, or literature review of the appropriate taxonomic level. Morphological images of the live nematodes isolated from I&Q SHB colony tubes and local PSHB colonies are shown in **Figure 3.2** and **Figure 3.3**, respectively.

Impact of nematode infestation on lab-reared PSHB colonies

Exposure to nematodes had no effect on colony establishment, and across all replicates, approximately two thirds of PSHB foundresses in the control and treatment groups established a successful colony ($\chi 2 = 0.0066$, df = 1, $p = 0.935$; **Figure 3.4**). Looking only at these successful colonies, negative binomial regression (**Table 3.3**) revealed that the introduction of nematodes had a strong negative effect ($P < 0.0001$) on the overall size of the PSHB colonies (all offspring, living and dead) (**Figure 3.5**), on the number of living offspring (**Figure 3.6**), and on the number of juvenile offspring (**Figure 3.7**). Dissection time point was also a significant factor in all three counts (**Table 3.3**), but without any clear pattern (**Figures 3.5 – 3.7**). Replicate was not a significant factor (**Table 3.3**). Across all time points, from 558 colonies that were initially inoculated with nematodes (5-week & 8-week), or derived from such colonies (8-week dispersed), living nematodes were not found in 315 (56%) of the tubes when the colony was dissected, suggesting that the nematodes died out or did not establish. Comparison of the number of living offspring in colonies with and without living nematodes revealed that the continued presence of living nematodes had a significant negative effect on offspring count, but time point did not (**Table 3.4**, **Figure 3.8**).

Observational notes during colony dissections

Among the beetles that visibly displayed signs of nematodes on their exoskeleton, the nematodes were consistently found in large numbers under the elytra, and formed a nematangia (pocket-like structure filled with nematodes) (**Figure 3.9**). In some cases, the entire surface of multiple larval instars along with male and female pupae were covered with a glossy, opaque film produced by large numbers of nematodes (**Figure 3.10**). In addition, some of the live adult beetles in tubes with high infestation of nematodes were found with damaged elytra, and hindered mobility as a result of mass accumulation of nematodes around the joints of the legs (**Figure 3.11**).

Discussion

Diversity of nematode species

The majority of nematodes found in bark and ambrosia beetle galleries are commonly dismissed as simply phoretic. However, biological interactions between ambrosia beetles and other organisms are often complex in forest ecosystems. In our study, we isolated 12 different nematode taxa, including species belonging to the families Aallantonematidae, Aphelenchoididae, and Diplogastridae. The substantial number of nematode species isolated from the *E. fornicatus* species complex suggests a more deceptively complex association and unique role these organisms may have with the beetles. Each family alone is comprised of both phoretic and parasitic nematodes, which presented difficulty in narrowing the ecological relationship between the isolated

nematodes and beetles of the *E. fornicatus* species complex without conducting further inquiry.

Exact or near identification of the isolated nematodes we tested lacked consistency across 28S, COI, and 18S genes in GenBank likely due to the scarcity of available nematode sequences online. COI sequences varied significantly compared to 28S and 18S sequences as a result of few mitochondrial genes being considered in nematode studies. 18S is considered too conserved at the species level to resolve new phylogenetic relationships among different families (Powers et al., 1986), while 28S sequences can be too conserved and unreliable to evaluate phylogenetic relationships in a diagnostic approach (Stock et al., 2001).

Of the uploaded sequences, only one had an exact match in the GenBank database with the nematode *Rhabditolaimus leuckarti*. This sequence, however, matched similarly to an undescribed *Rhabditolaimus* and *Myctolaimus* sp. While the combined sequences of all three genes resulted in order level taxon IDs, a few individual sequences from each gene require a closer look via additional sequencing to be certain of their ID. Among the isolated nematodes, the fungal-feeding nematode, *Bursaphelenchus penai*, is a commonly known phoretic nematode associated with *Xyleborus spp* (Kanzaki et al., 2014), but, prior to our study, it has never been reported with the *Euwallacea fornicatus* species complex.

Challenges with proper identification were not only faced using molecular sequences, but also with morphological characteristics. Not all morphological traits observed on the nematode species were in complete agreement with the feeding habits/life history of the species identified through sequence similarity. The encapsulated

pharynx in **Figures 3.2B** and **C** corresponds to the nematode genus *Myctolaimus*, yet the molecular sequences match, not only to the undescribed *Myctolaimus sp*., but also to *Rhabditolaimus leuckarti*. The use of DNA sequences for the reliable species identification of nematodes, will only be possible when systematic studies that combine morphological identification of nematodes with their DNA sequences have been done.

Potential to suppress invasive SHB populations

Although scolytine associations of nematodes have been surveyed because of their active role in controlling forest pests, the focus has rarely been on phoretic nematodes. Since phoretic species have limited mobility and greatly rely on the successful dispersal of their host to reach new habitats, it is commonly believed that these phoronts will not harm their host nor negatively impact their dispersal. For example, nematodes in the genus *Rhabditolaimus* are commonly thought to feed on bacteria, and use the beetle for phoretic transport. However, our laboratory trials show that the presence of a single nematode species, *Rhabditolaimus* sp., leads to a reduction in total offspring, living offspring, and juvenile offspring. Perhaps this more complex interaction is because there is a close association between SHBs and nematodes within the galleries.

It is unclear how these laboratory data can be translated to the field and should be viewed within the context of the study. For instance, as the number of nematodes gradually increased, the environmental conditions of the artificial diets likely changed. While an increase in humidity would commonly be favorable for the maintenance of the beetle's symbiotic fungi, the excess buildup of moisture, along with the scarce ventilation, may have facilitated the degradation of the artificial diet in the nematode

treated tubes. This was more evident as higher mortality of adult and immature stages of PSHB beetles were commonly observed during the 8-week dissections than in the 5-week dissections. In particular, a number of larvae and pupae would be observed on the surface of the colony tubes 5 weeks post nematode introduction, only to be found dead at the 8-week mark (**Figure 3.12**). These factors may have also accelerated the growth of fungal contaminations that were occasionally observed in the study. Another challenge faced during this study were phoretic mites (*Histiogaster arborsignis* Berlese), which likely impacted beetle mortality in both the control and nematode treatment groups. In a few instances, large populations of both nematodes and mites enveloped larval, pupal, and adult beetle carcasses (**Figure 3.13)**. While the frequency of mite occurrence in the laboratory trials was low, their interaction with nematodes and shothole borers is possibly more frequent and complex in the field. Low mortality rate of carrion beetles was observed when phoretic mites are present in broods, whereas larger population densities of nematodes and lower survivability of beetles was reported during the absence of phoretic mites (Wilson & Knollengberg, 1987).

While mortality and fitness costs were found in this study, the behavior of *Rhabditolaimus* is also consistent with simple phoresy. Only 44% of the total number of colonies initially inoculated with nematodes, supported a visible and viable nematode population at the time of dissection. Also, the presence of *Rhabditolaimus* did not affect the establishment of a colony by female adult beetles. The majority of nematodes observed in the galleries demonstrated an upright, side-to-side motion (a tactic commonly known as nictation) to latch onto hosts (Brown, 2011). While nematode and mite

populations can create an unfavorable environment for the beetles in colony tubes, this may not be the case in the field as a result of the grooming behavior executed by adult females. Grooming behavior of beetle foundresses involves the removal of excess frass in galleries and, in doing so, would shuffle out sick, infected, or dead offspring (Wichmann, 1967). In nature, the frass, and anything else (i.e. infected individuals) is free to fall to the floor and is effectively removed from the vicinity of the gallery. In artificial diet tubes, the potentially infected or sick individuals are shuffled out of the gallery only to pile up on the surface immediately adjacent to the gallery entrance (**Figure 3.13)**. This might lead to re-infestation of the gallery and maintain a nematode population in the tube.

Among ambrosia beetles, the *Rhabditolaimus* nematode species appears to be specifically associated with *E. fornicatus* beetles as the same nematode sequence found in Taiwan was isolated in Vietnam, and has been detected at least two sites in southern California. Could these nematodes have played a role in the decline in SHB populations that has been documented in several areas in California (Boland, 2016; Bennett, 2020)? Unfortunately no data have been collected on the presence of nematodes in these populations. Consequently it remains unclear and should be studied.

While the adverse effects observed in the laboratory experiments are promising, there are multiple factors that limit the use of nematodes as biological control agents. The most common limitation involves abiotic factors such as lack of moist environments and high temperatures (Glazer, 2002). Nematodes require free water for mobility and heavily depend on the successful dispersal of their host. Also, a considerable factor is competition for viable hosts with biological control agents (e.g., parasitoid wasps).

Survival of parasitoid larvae will likely be compromised in the presence of parasitic nematode species as the host longevity will deteriorate prior to full development of the parasitoid wasp. Studies report lower success rate of parasitism in newly emerged, naïve braconid wasps in comparison to mature, older parasitoids as a result of lack of experience in differentiating nematode-infested vs. non-infested hosts (Harvey et al., 2012). That said, while parasitoids of shothole borers in the *E. fornicatus* species complex have been identified, the challenge of rearing these species still remains after seven years of trial and error. Our limited understanding of this complex community is apparent and requires a thorough inquiry behind the multitrophic interactions to better assess true fitness costs and benefits under natural settings. In conclusion, we report the first case of a phoretic nematode with costly effects on PSHB populations in a laboratory setting. Further investigation in a field experiment is required to properly assess the true relationship between *Rhabditolaimus* nematodes and PSHB beetles.

References

Alizon, S. (2008). Transmission-recovery trade-offs to study parasite evolution. *The American Naturalist, 172*(3), E113-E121.

Allard, G. B., & Moore, D. (1989). *Heterorhabditis* sp. nematodes as control agents for Coffee Berry Borer, *Hypothenemus hampei* (Scolytidae). *Journal of Invertebrate Pathology, 54*(1), 45-48.

Ashraf, M., & Berryman, A.A. (1970). Biology of *Sulphuretylenchus elongatus* (Nematoda: Spaerulariidae) and its effect on its host *Scolytus ventralis* (Coleoptera: Scolytidae). *Canadian Entomologist, 102*, 197-213.

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

Bennett, S. K. (2020). *Ecology of the Invasive Shot Hole Borer (Euwallacea whitfordiodendrus) in a Coastal California Riparian System*. University of California, Santa Barbara.

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

Browne, F.G. (1961), The biology of Malayan Scolytidae and Platypodidae. *Malayan Forest Records, 22*, 1– 255.

Cardoza, Y. J., Paskewitz, S., & Raffa, K. F. (2006). Travelling through time and space on wings of Beetles: A Tripartite insect-fungi-nematode association. *Symbiosis, 41*, 71-79.

Carrillo, J. D., Dodge, C., Stouthamer, R., & Eskalen, A. (2020). Fungal symbionts of the polyphagous and Kuroshio shot hole borers (Coleoptera: Scolytinae, *Euwallacea spp*.) in California can support both ambrosia beetle systems on artificial media. *Symbiosis, 80*(2), 155-168.

Castillo, A., Infante, F., Barrera, J. F., Carta, L., & Vega, F. E. (2002). First field report of a nematode (Tylenchida: Sphaerularioidea) attacking the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) in the Americas. Journal of Invertebrate Pathology, *79*, 199-202.

Eatough Jones, M., & 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.

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 Disease*, *97*(7), 938-951.

Floyd, R. M., Rogers, A. D., Lambshead, P. J. D., & Smith, C. R. (2005). Nematode-specific PCR primers for the 18S small subunit rRNA gene. *Molecular Ecology Notes*, *5*(3), 611-612.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular marine biology and biotechnology*, *3*(5), 294–299.

Furniss, M. M. (1967). Nematode parasites of the Douglas-fir beetle in Idaho and Utah. *Journal of Economic Entomology*, *60*(5), 1323-1326.

Giblin-Davis, R. M., Kanzaki, N., & Davies, K. A. (2013). Nematodes that Ride Insects: Unforeseen Consequences of Arriving Species. *Florida Entomologist*, *96*(3), 770-780.

Glazer, I. (2002). Survival biology. Entomopathogenic Nematology. *Ed.: Gaugler, R., CABI, Wallinford, Oxon, UK*, 167-187.

Hall, T. A. (1999). BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. Nucleic Acids Symposium Series, *41*, 95-98.

Harvey, C. D., & Griffin, C. T. (2012). Host activity and wasp experience affect parasitoid wasp foraging behaviour and oviposition on nematode-infected larvae of the forestry pest *Hylobius abietis*. *Ecological Entomology*, *37*(4), 269-282.

Hoffard, W. H., & Coster, J. E. (1976). Endoparasitic Nematodes of *Ips* Bark Beetles in Eastern Texas. *Environmental Entomology*, *5*(1), 128-132(5).

Hulcr, J., Black, A., Prior, K., Chen, C. Y., & Li, H. F. (2017). Studies of ambrosia beetles (Coleoptera: Curculionidae) in their native ranges help predict invasion impact. *Florida Entomologist*, *100*(2), 257-261.

Hunt, D. J., & Hague, N. G. M. (1974). A Redescription of *Parasitaphelenchus oldami* Ruhm, 1956 (Nematoda: Aphelencjhoididae) a Parasite of Two Elm Bark Beetles: *Scolytus scolytus* and *S. multistriatus*, together with some notes on its biology. *Nematologica*, *20*(2), 174-180.

Kanzaki, N., Aikawa, T., Maehara, N., & Ichihara, Y. (2011). An inoculation experiment of Japanese Bursaphelenchus nematodes on Japanese black and red pine, *Pinus thunbergii* and *P. densiflora*. *Journal of forest research*, *16*(4), 325-330.

Kanzaki, N., Masuya, H., Taki, H., Okabe, K., & Chen, C. Y. (2013). Description of *Ruehmaphelenchus formosanus* n. sp.(Tylenchina: Aphelenchoididae) isolated from *Euwallacea fornicates* from Taiwan. *Nematology*, *15*(8), 895-906.

Kanzaki, N., Giblin-Davis, R. M., Carrillo, D., Duncan, R., & Gonzalez, R. (2014). *Bursaphelenchus penai* n. sp.(Tylenchomorpha: Aphelenchoididae), a phoretic associate of ambrosia beetles (Coleoptera: Scolytinae) from avocado in Florida. *Nematology*, *16*(6), 683-693.

Kaya, H. K., & Moon, R. D. (1978). The nematode *Heterotylenchus autumnalis* and face fly *Musca autumnalis*: a field study in northern California. *Journal of Nematology*, *10*(4), 333.

Kaya, H. K., & Hotchkin, P. G. (1981). The nematode *Neoaplectana carpocapsae* Weiser and its effect on selected ichneumonid and braconid parasites. *Environmental entomology*, *10*(4), 474-478.

Kaya, H. K., & Stock, S. P. (1997). Techniques in insect Nematology. In Manual of techniques in insect pathology: Biological Techniques Series.

Little, T. J., Chadwick, W., & Watt, K. (2008). Parasite variation and the evolution of virulence in a Daphnia-microparasite system. *Parasitology*, *135*(3), 303-308.

Liu, J., Berry, R. E., & Moldenke, A. F. (1997). Phylogenetic relationships of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) inferred from partial 18S rRNA gene sequences. *Journal of Invertebrate Pathology*, *69*(3), 246-252.

Massey, C. L. (1960). Nematode parasites and associates of the California five-spined engraver, *Ips confusus* (Lec.). *Proceedings of The Hemlmitological Society of Washington*, *27*(1), 42-44.

Massey, C. L. (1964). The nematode parasites and associates of the fir engraver beetle, *Scolytus ventralis* Le Conte, in New Mexico. *Journal of Insect Pathology*, *6*(2), 133-155.

Massey, C. L. (1966). The nematode parasites and associates of Dendroctonus adjunctus (Coleoptera: Scolytidae) in New Mexico. *Annals of the Entomological Society of America*, *59*(3), 424-440.

Massey, C. L. (1974). Biology and taxonomy of nematode parasites and associates of bark beetles in the United States. Agricultural Handbook No. 446. *United States Department of Agriculture. Washington DC, USA*.

Mendel, Z., Protasov, A., Sharon, M., Zveibil, A. Y. S. B., 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*(3), 235-238.

Moser, J. C., Konrad, H., Kirisits, T., & Carta, K. L. (2005). Phoretic mites and nematodes associates of *Scolytus multistriatus* and *Scolytus pygmeus* (Coleoptera: Scolytidae) in Austria. *Agricultural and Forest Entomology*, *7*(2), 169-177.

Nermuť, J., Půža, V., & Mráček, Z. (2012). Entomopatogenní a moluskoparazitické hlístice–neviditelní půdní zabijáci. *Živa*, *1*, 10-12.

Nickle, W. R. (1963). Observations on the effect of nematodes on *Ips confusus* (Le Come) and other bark beetles. *Journal of Insect Pathology*, *5*(3), 386-389.

Paap, T., De Beer, Z. W., Migliorini, D., Nel, W. J., & 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.

Poinar, G. O. (1975). Entomogenous Nematodes: A Manual and Host List of Insect-nematode Associations. Leiden, Netherlands (E. J. Brill). 317 p.

Powers, T. O., Platzer, E. G., & Hyman, B. C. (1986). Species-Specific Restriction Site Polymorphism in Root-knot Nematode Mitochondrial DNA. *Journal of nematology*, *18*(3), 288–293.

Reid, R. W. (1958). Nematodes associated with the mountain pine beetle. *Bimonthly Progress Report, Canada Department of Agriculture*, *14*(1), 3.

Rugman-Jones, P. F., Hoddle, M. S., & Stouthamer, R. (2010). Nuclear-mitochondrial barcoding exposes the global pest western flower thrips (Thysanoptera: Thripidae) as two sympatric cryptic species in its native California. *Journal of economic entomology*, *103*(3), 877-886.

Ruhm, W. (1954). A new nematodes species of *Ips* bark beetles. *Zoologischer Anzeiger Leipzig*, *53*(9-10), 221-242.

Ruhm, W. (1955). About some nematode species associate of wood breeding Ips bark beetles. *Zoologischer Anzeiger Leipzig*, *155*(3-4), 70-83.

Ruhm, W. (1956). The Nematodes of Bark Beetles. *Parasitologische Schriftenreihe*, *6*, 1-437.

Patricia Stock, S., Campbell, J. F., & Nadler, S. A. (2001). Phylogeny of *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *Journal of Parasitology*, *87*(4), 877-889.

Stouthamer, R., Rugman-Jones, P., Thu, P. Q., Eskalen, A., Thibault, T., Hulcr, J., Wang, L., Jordal, B. H., Chen, C., Cooperband, M., Ling, C., Kamata, N., Lu, S., Masuya, H., Mendel, Z., Rabaglia, R., Sanguansub, S., HsinHui, S., Sittichaya, W., & Zong, S. (2017). Tracing the origin of a cryptic invader: phylogeography of the *Euwallacea fornicatus* (Coleoptera: Curculionidae: Scolytinae) species complex. *Agricultural and Forest Entomology*, *19*(4), 366-375.

Takov, D., Pilarska, D., & Wegensteiner, R. (2006). Entomopathogens in *Ips typographus* (Coleoptera, Scotylidae) from several spruce stands in Bulgaria. *Acta Zoologica Bulgarica, 58*, 409-420.

Thong, C. H. S., & Webster, J. M. (1983). Morphology and the postembryonic development of the bark beetle nematode *Contortylenchus reversus* (Sphaerularidae). *Nematologica*, *19*(2), 159-168.

Truett, G. E., Heeger, P., Mynatt, R. L., Truett, A. A., Walker, J. A., & Warman, M. L. (2000). Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques*, *29*(1), 52-54.

Wang, Y., & Rozen, D. E. (2019). Fitness costs of phoretic nematodes in the burying beetle, *Nicrophorus vespilloides*. *Ecology and Evolution*, *9*(1), 26-35.

Weber, B. C., & McPherson, J. E. (1983). Life history of the ambrosia beetle *Xylosandrus germanus* (Coleoptera: Scolytidae). *Annals of the Entomological Society of America*, *76*(3), 455-462.

Weiser, J., Holusa, J., & Zizka, Z. (2006). *Larssoniella duplicati* n. sp. (Microsporidia, Unikaryonidae), a newly described pathogen infecting the double-spined spruce bark beetle, *Ips duplicatus* (Coleoptera, Scolytidae) in the Czech Republic. *Journal of Pest Science*, *79*, 127-135.

White, P. S., Morran, L., & de Roode, J. (2017). Phoresy. *Current Biology*, *27*(12), R578-R580.

Wichmann, H. E. (1967). Die Wirkungsbreite des Ausstoβreflexes bei Borkenkäfernreflexes bei Borkenkäfern. *Anzeiger für Schädlingskunde*, *40*(12), 184-187.

Wood, S. L., & Bright Jr, D. E. (1992). Index for Scolytidae. *Great Basin Naturalist Memoirs*, *13*(1), 16.

Zaki, F. N., Awadallah, K. T., & Gesraha, M. A. (1997). Competitive interaction between the braconid parasitoid, *Meteorus rubens* Nees and the entomogenous nematode, *Steinernema carpocapsae* (Weiser) on larvae of *Agrotis ipsilon* Hufn. (Lep., Noctuidae). *Journal of Applied Entomology*, *121*(1-5), 151-153.

Figure 3.1. Polyphagous shothole borer (PSHB) artificial diet tube with visible signs of nematode infestation.

Figure 3.2. Compiled images of Rhabditida spp isolated from beetle-artificial diet media. The circled sections (**3.2B, 3.2C)** indicate the presence of an encapsulated pharynx. The mature beetles introduced to the diet media with signs of nematodes emerged from castor bean and avocado infested wood material originally collected at three different sites in Taiwan: CB1 (Rhabditida sp.1, **3.2A**), CB2 (Rhabditida sp.3, **3.2B**), and Y1 (Rhabditida sp.4, **3.2C**).

Figure 3.3. *Rhabditolaimus* (= *Myctolaimus*) sp.1 isolated from local established PSHB populations in southern California.

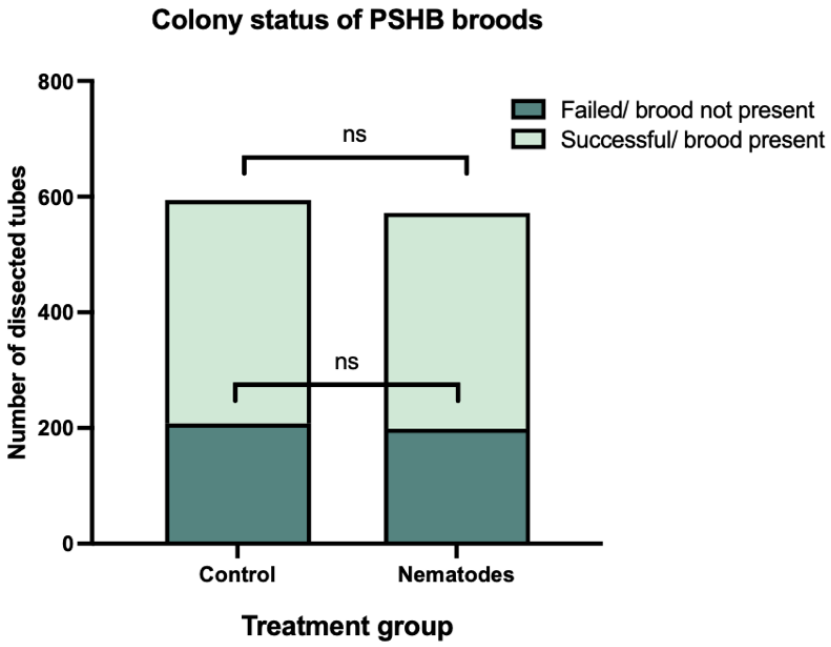

Figure 3.4. Chi-square test comparison of number of failed vs successful PSHB colonies in the presence (nematode) and absence (control) of *Rhabditolaimus* nematode species. There was no significance ($P = 0.935$) in the observed number of failed and successful beetle colonies across both treatment groups ($\chi 2 = 0.0066$, df = 1).

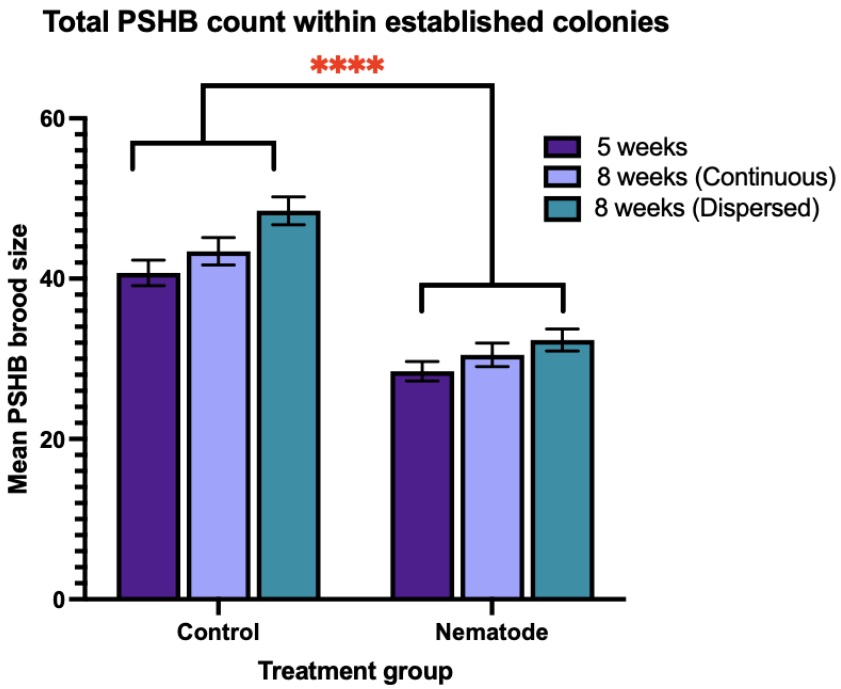

Figure 3.5. Mean total (viable and dead) PSHB count in both control and nematode treatment groups. Total count is compiled from each dissection time point: 5 weeks, 8 weeks that represent a continuous offspring generation, and 8 weeks that represent a dispersed F1 generation collected from the 5-week mark to start a colony in a new artificial diet tube. Negative binomial regression analysis was performed and revealed that the presence of nematodes significantly affected the mean total PSHB count in the nematode group in comparison to the control ($P < 0.0001$).

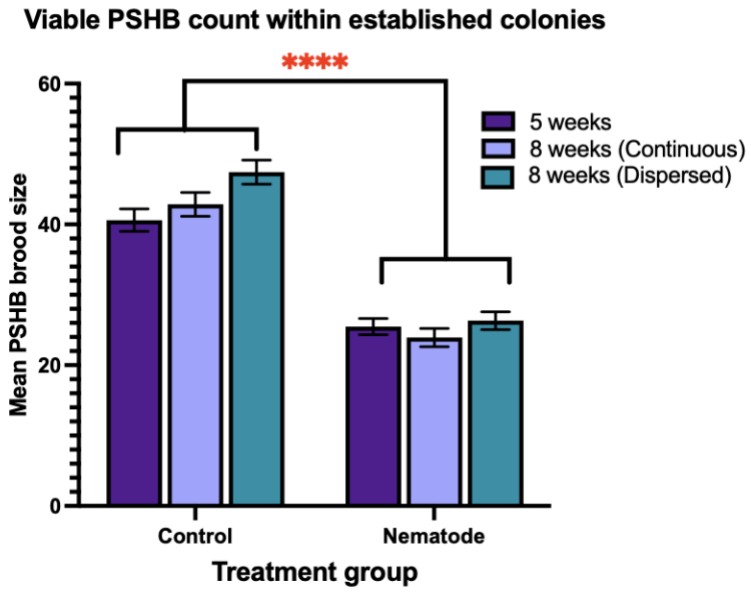

Figure 3.6. Mean count of viable PSHB life stages in both control and nematode treatment groups. Viable PSHB count is compiled from each dissection time point: 5 weeks, 8 weeks that represent a continuous offspring generation, and 8 weeks that represent a dispersed F1 generation collected from the 5-week mark to start a colony in a new artificial diet tube. Negative binomial regression analysis revealed the presence of nematodes significantly affected the viable count in the nematode group in comparison to the control ($P < 0.0001$).

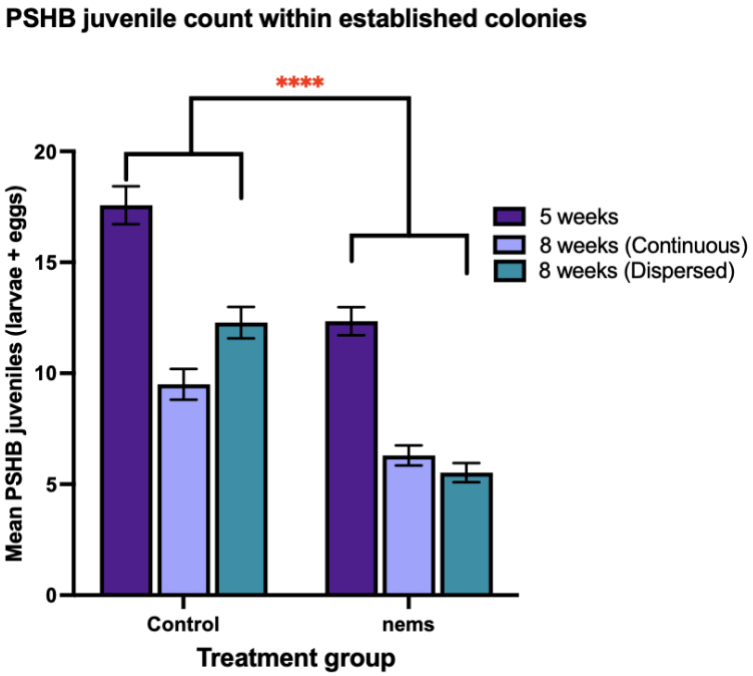

Figure 3.7. Mean count of PSHB juveniles (larvae and eggs) in both control and nematode treatment groups. The mean count of juveniles is compiled from each dissection time point: 5 weeks, 8 weeks that represent a continuous offspring generation, and 8 weeks that represent a dispersed F1 generation collected from the 5-week mark to start a colony in a new artificial diet tube. Negative binomial regression analysis revealed the presence of nematodes significantly affected the mean count of juveniles in the control and nematode treatment groups ($P < 0.0001$).

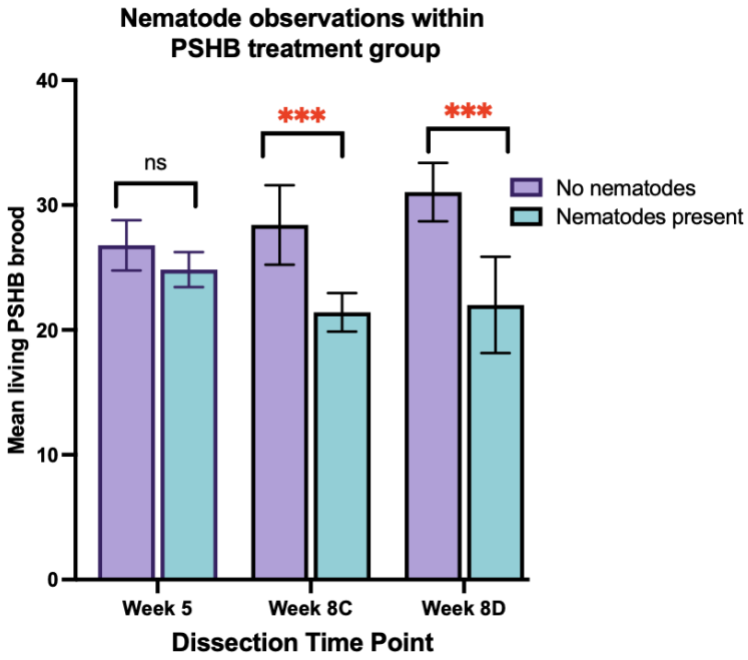

Figure 3.8. Pairwise comparison using ANOVA analysis to determine means of PSHB colony within the nematode treatment group. Colonies with visible signs of nematodes were compared to those that did not quantify visible nematode numbers. Mean viable PSHB count in colonies absent of nematodes were significantly higher ($P < 0.0001$) than colonies that quantified visible nematodes, with the exception of week 5 dissections.

Figure 3.9. Nematangia formed on the elytra of PSHB beetle.

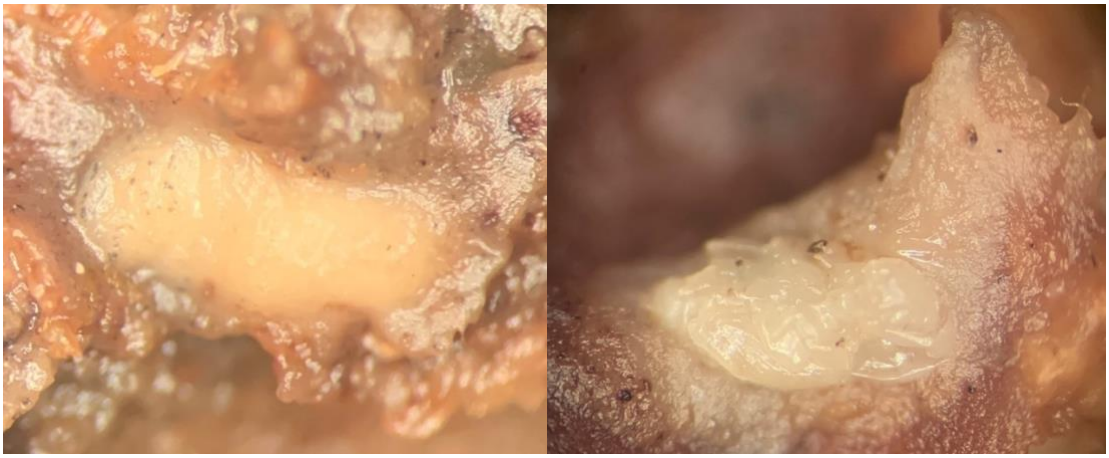

Figure 3.10. Images taken 8 weeks post nematode introduction of a dead 3rd instar larva (**Left**) and a dead female pupa (**Right**). Both the dead larva and pupa are completely covered and surrounded by nematodes.

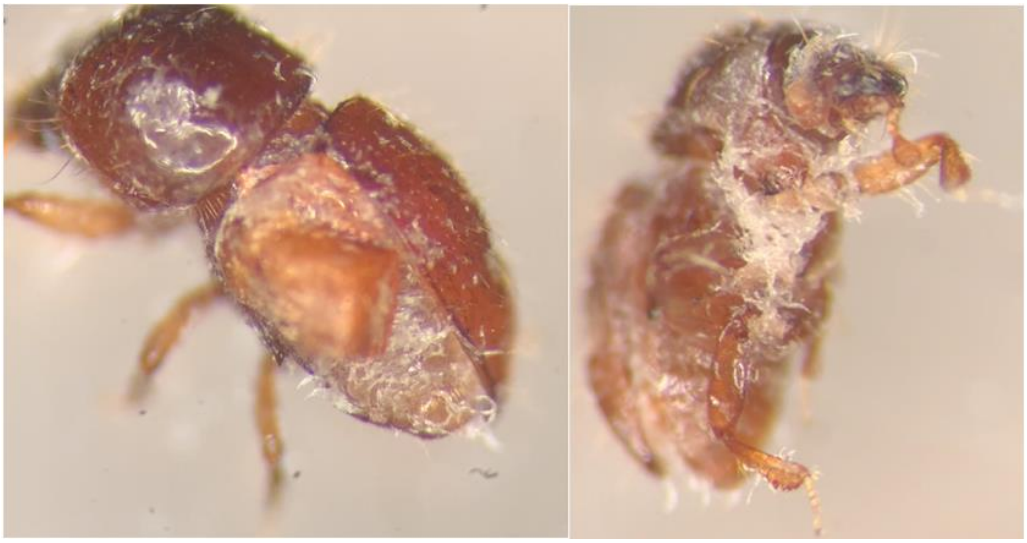

Figure 3.11. Dorsal and ventral images taken of a teneral male PSHB adult with a damaged/underdeveloped elytra and is heavily infested with nematodes.

Figure 3.12. Before and after images taken of a PSHB tube inoculated with nematodes. **Left**: image taken 5 weeks post nematode introduction. **Right**: image taken of the same tube 8 weeks post nematode introduction followed by gallery dissection for offspring count data. While the larvae and pupae were able to complete their development to adults, the teneral and mature female and male adults were found dead and covered in nematodes.

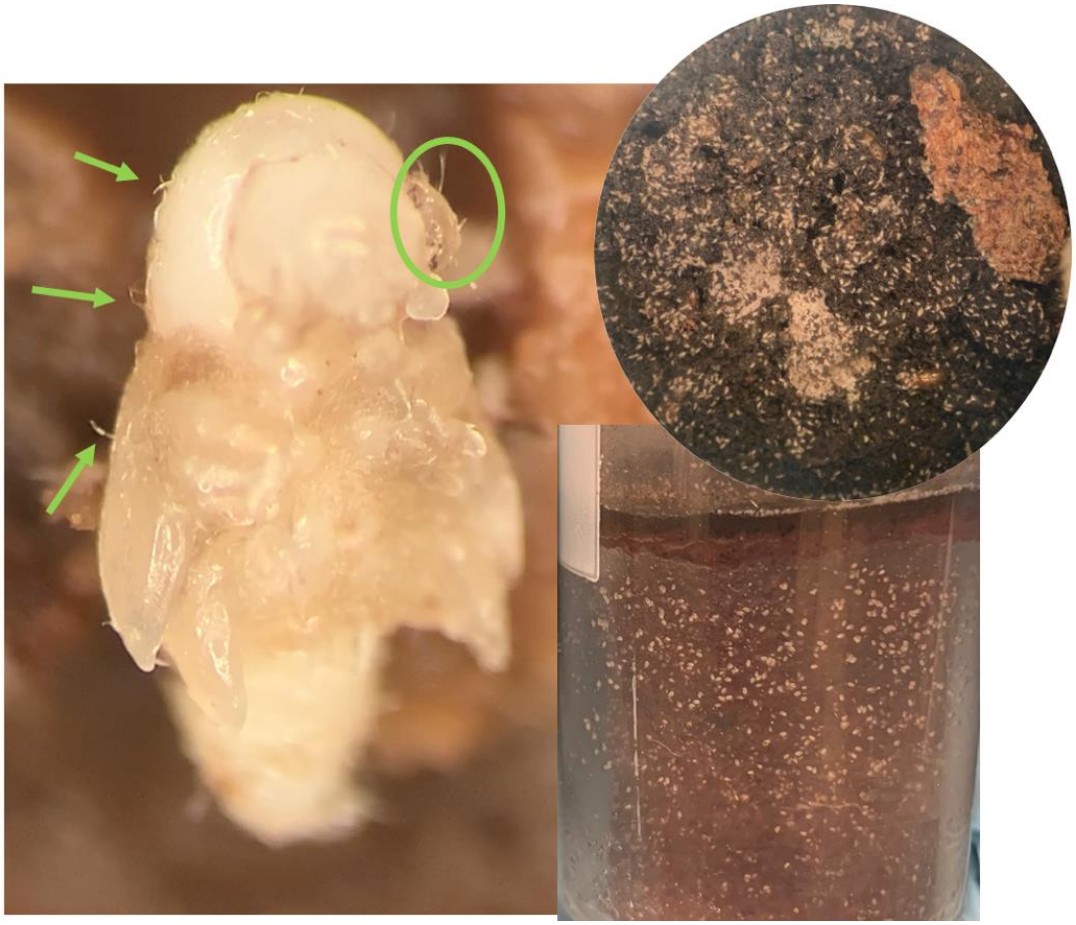

Figure 3.13. PSHB artificial diet tube that was initially inoculated with nematodes and later discovered to be infested with mites. The mites are visible on the top surface and sides of the tube as well as inside the beetle gallery. Tubes treated with nematodes that were susceptible to mite infestation resulted in 100% mortality.

Table 3.1. Collecting information for the shothole borer infested wood, from which nematodes were subsequently isolated. Sites are all in Danei, Taiwan, and information includes GPS coordinates, wood species, and the identity of the beetles that emerged from that wood; PSHB = polyphagous shothole borer, TSHB = tea shothole borer, KSHB = Kuroshio shothole borer. H22 likely represents a fourth species within the *E. fornicatus* species complex (Stouthamer et al. 2017).

Collection site	Coordinates	Host plant	H22	PSHB (H38)	TSHB (H8)	KSHB (H20)
CB1	23.103076, 120.376758	Castor bean	22	0	0	0
CB2	23.103838, 120.370365	Castor bean	25	0	0	0
CB3	23.109410, 120.384017	Castor bean	28	0	0	0
AC4	23.103128, 120.376497	Avocado	0	31	9	0
Y1	23.110714, 120.378915	Avocado	9	14	0	0
Y2	23.109904, 120.369750	Avocado/Castor bean	0	23	5	0
L1	23.140445, 120.403233	Avocado	0	19	4	0
L2	23.139684, 120.403491	Avocado	0	21	3	0
L3	23.144393, 120.404804	Avocado	0	36	11	1
L4	23.143261, 120.401374	Avocado	0	17	0	0

Table 3.2. Molecular identification of nematodes isolated directly from beetles in the *Euwallacea fornicatus* species complex collected from imported native wood material, and from artificial media used to rear beetles from Taiwan and southern California. Closest match ID of nematode species was attempted using 28S, 18S, and COI gene sequences.

Taxon #	Nematode closest match ID	Level	GenBank Accessions			Relationship of association	Isolation source
			28S	18S	COI		
1	*Rhabditolaimus* (= *Myctolaimus*) sp.1	Genus	x	x	x	Phoretic/ parasitic	PSHB, TSHB
2	Rhabditida sp.1	Order	x		x	Phoretic/ parasitic	PSHB
3	*Aphelenchoides* sp.1	Genus	x		x	Parasitic	
4	Rhabditida sp.2	Order	x		x	Phoretic/ parasitic	
5	*Bursaphelenchus penai*	Species	x	x	x	Phoretic/ fungal-feeder	
6	Allantonematidae sp.1	Family		x		Phoretic/parasitic	
7	Rhabditida sp.3	Order			x	Phoretic/parasitic	PSHB
8	Rhabditida sp.4	Order			x	Phoretic/parasitic	PSHB
9	Rhabditida sp.5	Order			x	Phoretic/parasitic	PSHB
10	Rhabditida sp.6	Order			x	Phoretic/parasitic	PSHB
11	Rhabditida sp.7	Order			x	Phoretic/parasitic	PSHB
12	*Bursaphelenchus* sp.1	Genus			x	Phoretic/ fungal-feeder	

Table 3.3. Negative binomial regression (NBR) analysis of polyphagous shothole borer (PSHB) count data. NBR compared the effects of nematodes and dissection time point had on the mean PSHB count across all replicates in the control vs. nematode treatment groups.

Independent variables	Mean PSHB count data											
	Total brood				Viable brood				Juvenile stages			
	IRR	Coef.	z	P>| z |	IRR	Coef.	z	P>| z |	IRR	Coef.	z	P>| z |
Nematode	0.688	-0.375	-10.15	0.0001	0.576	-0.551	-13.54	0.0001	0.572	-0.558	-8.74	0.0001
Time point	1.019	0.018	3.43	0.001	1.013	0.013	2.22	0.027[a]	0.942	-0.060	-6.65	0.0001
Experiment replicates	1.003	0.003	0.54	0.586	1.003	0.004	0.45	0.650	1.012	0.124	1.13	0.260

[a] slight marginal significance

Table 3.4. Two-way ANOVA comparing the number of living offspring in PSHB colonies that still support a viable nematode population at various time points following an initial inoculation with ~500 *Rhabditolaimus* sp.

	Partial SS	df	MS	F	p
Model	3817.21	3	1272.40	6.82	0.0002
Nematode (+/-)	3323.11	1	3323.11	17.82	<0.0001
Time point	235.99	2	118.00	0.63	0.5317
Residual	65079.47	349	186.47		
Total	68896.69	352	195.73		