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Biology of Two Invasive Ambrosia Beetles, the Polyphagous and Kuroshio Shot Hole Borers (*Euwallacea* spp.), in California

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

Doctor of Philosophy

in

Entomology

by

Christine Elizabeth Dodge

December 2019

Dissertation Committee:
Dr. Richard Stouthamer, Chairperson
Dr. Timothy Paine
Dr. Akif Eskalen

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The Dissertation of Christine Elizabeth Dodge is approved:

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Committee Chairperson

University of California, Riverside

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The text of this dissertation, in part, is a reprint of the material as it appears in "Quercivorol as a lure for the polyphagous and Kuroshio shot hole borers, *Euwallacea* spp. nr. *fornicatus* (Coleoptera: Scolytinae), vectors of Fusarium dieback" (2017). The co-author Jessica Coolidge listed in that publication assisted with data collection. The co-authors Miriam Cooperband, Allard Cossé, and Daniel Carrillo helped design the experiments. The co-author Richard Stouthamer directed and supervised the research which forms the basis for this dissertation.

The text of this dissertation, in part, is a reprint of the material as it appears in "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" (in press). The co-first author Joseph Carrillo listed in that publication

contributed equally to experimental design, data collection, and analysis. The co-authors Akif Eskalen and Richard Stouthamer directed and supervised the research.

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Dedication

In loving memory of the one, the only, D. M. F. J.

ABSTRACT OF THE DISSERTATION

Biology of Two Invasive Ambrosia Beetles, the Polyphagous and Kuroshio Shot Hole Borers (*Euwallacea* spp.), in California

by

Christine Elizabeth Dodge

Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, December 2019
Dr. Richard Stouthamer, Chairperson

The polyphagous shot hole borer (PSHB) *Euwallacea fornicatus* and the Kuroshio shot hole borer (KSHB) *Euwallacea kuroshio* are two invasive species of concern in Southern California. Both are ambrosia beetles, small, wood-boring weevils that farm and feed on fungi inside of host trees. PSHB and KSHB attack living and apparently healthy trees, and their fungi are phytopathogens that, together with boring damage from the beetles, are responsible for the plant disease Fusarium dieback. PSHB and KSHB were first discovered in California in 2003 and 2014, respectively, and until recently many aspects of their biology were unknown. The goal of this dissertation was to expand the body of knowledge pertaining to these two species in their invasive range in California, using both applied and fundamental research. In Chapter 1, I describe a series of field experiments that were performed to assess the use of various lures and repellents for PSHB and KSHB, as well as the effect of trap modifications on beetle capture. I find that quercivorol is a suitable lure for both species, and that both species are repelled by

verbenone and piperitone, which may prove useful in deterring beetle entry into naïve areas. In Chapter 2, I reared KSHB at a series of constant temperatures to determine its effect on their development and emergence. The upper and lower temperature thresholds for KSHB development were found to be about 32°C and 13°C, respectively, and optimal development occurs at about 28°C. I also predict the annual number of KSHB generations for invaded areas in California using climate data from the California Irrigation Management Information System. In Chapter 3, I performed experiments to determine if PSHB and KSHB could survive and reproduce on each other's fungal symbionts. Both beetles could subsist on the others' *Fusarium* symbiont, but reproduction was reduced on auxiliary *Graphium* and *Paracremonium* fungi, as well as non-ambrosial *Fusarium* species. Finally, in Chapter 4, I perform population genetic analysis of California populations of PSHB and KSHB to measure genetic diversity, admixture, and outbreeding. Rates of outbreeding and heterozygosity were higher than expected under the accepted hypothesis of strict sibling mating.

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INTRODUCTION

Agriculture in the United States is under continuous threat from invasive species, particularly phytophagous insects, which are important vectors of plant disease and are additionally capable of causing substantial mechanical damage to crops. Wood-boring beetles are among the most common groups of invasive insects in North America, with potential to cause the most damage (Haack 2003, 2006). The polyphagous shot hole borer (PSHB) *Euwallacea fornicatus* Eichhoff and the Kuroshio shot hole borer (KSHB) *Euwallacea kuroshio* Gomez & Hulcr (Coleoptera: Curculionidae: Scolytinae) are two species of ambrosia beetle native to Southeast Asia (Stouthamer et al. 2017, Gomez et al. 2018, Smith et al. 2019). Invasive in Southern California, these shot hole borers (SHB) vector distinct fungal symbionts in the genus *Fusarium* (Ascomycota: Hypocreales), which, paired with mechanical damage from beetle boring activity, causes the plant disease Fusarium dieback in over 60 susceptible host tree species (Eskalen et al. 2013).

Characteristic of the ambrosia beetles, adult and larval stages of PSHB and KSHB are reliant upon their symbiotic fungi as a food source (Beaver 1989). Ambrosia beetles cultivate and manage fungal gardens – "ambrosia" – within their brood galleries, one of only four known cases of the evolution of agriculture in the animal kingdom (Biedermann et al. 2009). Before dispersal, adult females collect fungal spores in specialized glandular organs called mycangia, allowing them to introduce their symbiotic fungi to new hosts when they bore galleries of their own (Francke-Grosmann 1956, Farris 1963). Most ambrosia beetles colonize only stressed or dying trees, and thus are not considered pests in forest landscapes (Paine et al. 1997). However, some ambrosia

beetles, such as the polyphagous and Kuroshio shot hole borers, attack live and healthy trees, and their symbiotic fungi infest host tree vascular tissue. These phytopathogenic fungi utilize host nutrients to grow and block transport through the tree xylem, causing branch dieback and, in susceptible hosts, tree death (Eskalen et al. 2012).

Like all other members of the tribe Xyleborini, PSHB and KSHB are haplodiploid inbreeders (Normark et al. 1999, Farrell et al. 2001) and their broods exhibit extremely female-biased sex ratios (Kirkendall 1993). Adult female SHB mate with their brothers inside their natal gallery, ensuring fertilization prior to dispersal (Kirkendall 1983). However, fertilization is not a requirement; due to haplodiploid sex determination, an unmated female can produce sons from unfertilized eggs, which she can then mate with to produce daughters (Normark et al. 1999, Cooperband et al. 2016). Together with their habit of fungus farming, this provides them an extraordinary ability to colonize naïve habitats (Kirkendall and Jordal 2006). Female SHB typically engage in one short dispersal flight and are reported to be weak fliers (Gadd 1941, Cranham 1963). However, they are capable of long distance dispersal via wind currents (Judenko 1958, Calnaido 1965). Male SHB are small and flightless, and thus do not readily disperse (Judenko 1958). Apart from their brief dispersal period, SHB spend their lives protected within their host tree. The combined effects of the above factors provide extremely limited opportunities for SHB control.

SHB and the associated plant disease Fusarium dieback are rapidly spreading throughout Southern California. PSHB was first discovered in Southern California in 2003 (Rabaglia et al. 2006), but symptoms of Fusarium dieback did not appear until 2012

(Eskalen et al. 2012). Until this point, PSHB was mistaken for the tea shot hole borer (TSHB) *Euwallacea perbrevis* Schedl, a closely related species from which it is morphologically indistinguishable (Smith et al. 2019). *E. perbrevis* is a major pest of tea in Sri Lanka (Danthanarayana 1968, Walgama and Pallemulla 2005) and is invasive in the United States in Florida and Hawaii (O'Donnell et al. 2015, Stouthamer et al. 2017). *E. perbrevis* diverges significantly from what is now known as PSHB in the mitochondrial COI gene (Eskalen et al. 2013, Rugman-Jones and Stouthamer 2016, Stouthamer et al. 2017, Gomez et al. 2018). When KSHB was discovered in Southern California in 2014, it was mistaken for PSHB. Again, they were distinguished on the basis of COI divergence (Stouthamer et al. 2017, Gomez et al. 2018). Thus, the PSHB and KSHB were given their own common names. DNA barcoding of specimens from California, Israel, Asia, and Florida now gives evidence for at least four different species of shot hole borer, together forming a cryptic species complex (O'Donnell et al. 2015, Stouthamer et al. 2017). The various members of the *Euwallacea fornicatus* species complex also harbor different species of fungal symbionts (Kasson et al. 2013, O'Donnell et al. 2015), which vary in host preference and pathogenicity (Mendel et al. 2012, Eskalen et al. 2013, Carrillo et al. 2015, Lynch et al. 2016, Na et al. 2018). The following studies focus on just two members of this species complex, but have ecological implications for all of them.

Current preferred hosts of the beetle-fungus complex in Southern California include box elder, castor bean, and California sycamore, which are all common in urban landscaping, as well as willows, oaks, and poplars in natural riparian areas (Eskalen et al.

2013, Boland 2016). Another preferred host is avocado, an economically important crop in California; in 2010, avocados were California's fifth most valuable commodity and comprised a $460 million industry (Vergati and Sumner 2012). KSHB are established in commercial avocado groves in San Diego County, which has the highest avocado production in the state (Vergati and Sumner 2012). It is also present in natural areas in Orange County, and has been collected more recently farther north in Santa Barbara and San Luis Obispo Counties (Eskalen 2019b). PSHB is established in commercial avocado groves, natural areas, and urban landscapes in Orange, Los Angeles, and Ventura Counties in California (Eskalen 2019b). PSHB is also invasive in other areas worldwide, including Mexico, Israel, and South Africa (Mendel et al. 2012, García-Avila et al. 2016, Paap et al. 2018), where it has been reported to cause Fusarium dieback. There are currently no adequate control measures for the polyphagous and Kuroshio shot hole borers or their fungal pathogens.

Chapter I. Field evaluations of lures and repellents for the polyphagous and Kuroshio shot hole borers, *Euwallacea* spp. nr. *fornicatus* (Coleoptera: Scolytinae), vectors of Fusarium dieback

ABSTRACT

The polyphagous shot hole borer and Kuroshio shot hole borer, two members of the *Euwallacea fornicatus* species complex, are invasive ambrosia beetles that harbor distinct species of phytopathogenic *Fusarium* fungal symbionts. Together with structural damage caused by beetle gallery construction, these two *Fusarium* species are responsible for the emerging tree disease Fusarium dieback, which affects over fifty common tree species in Southern California. Host trees suffer branch dieback as the xylem is blocked by invading beetles and fungi, forcing the costly removal of dead and dying trees in urban areas. The beetles also threaten natural riparian habitats, and commercial avocado is susceptible as well, resulting in damage to the avocado industries in California and Israel. Currently there are no adequate control mechanisms for shot hole borers. This chapter summarizes efforts to find a suitable lure to monitor shot hole borer invasions and dispersal. Field trials were conducted in two counties in Southern California over a span of two years. We find that the chemical quercivorol is highly attractive to these beetles, and perform subsequent field experiments attempting to optimize this lure. We also explore other methods of increasing trap catch and effects of other potential attractants, as well as the deterrents verbenone and piperitone.

INTRODUCTION

Fusarium dieback is an emerging plant disease, first reported in Israel in 2009 (Mendel et al. 2012) and in Southern California in 2012 (Eskalen et al. 2012). The disease is caused in part by two plant pathogenic fungi in the genus *Fusarium* (Ascomycota: Hypocreales), each of which is associated with an ambrosia beetle in the *Euwallacea fornicatus* cryptic species complex (Coleoptera: Curculionidae: Scolytinae) (Kasson et al. 2013, O'Donnell et al. 2015, Cooperband et al. 2016, Stouthamer et al. 2017). This species complex consists of at least three, and possibly five, morphologically indistinguishable ambrosia beetles from Southeast Asia (Stouthamer et al. 2017). Three members of this complex have invaded the United States: two in Southern California, and one in Florida and Hawaii. Until recently all members were thought to be the tea shot hole borer (TSHB) *Euwallacea perbrevis* Schedl (Smith et al. 2019, formerly recognized as *E. fornicatus*), a serious pest of tea in Sri Lanka (Austin 1956, Walgama and Pallemulla 2005). Although morphologically indistinguishable, molecular analyses revealed significant divergence in mitochondrial and nuclear genes of all three beetles (Eskalen et al. 2013, Stouthamer et al. 2017), which were subsequently given different common names to distinguish them. The beetle clade invading Florida and Hawaii is thought to be *Euwallacea perbrevis* (TSHB clade a), and is referred to here as the TSHB (Stouthamer et al. 2017). Two distinct invasions occurred in Southern California: the beetle that invaded the Los Angeles area is *E. fornicatus* Schedl, the polyphagous shot hole borer (PSHB; Eskalen et al. 2013, Cooperband et al. 2016). The species that invaded the San Diego area is *E. kuroshio* Gomez & Hulcr, the Kuroshio shot hole borer (KSHB;

Stouthamer et al. 2017). Each beetle has a different *Fusarium* symbiont, and all symbionts associated with these shot hole borers (SHB) are members of the Ambrosia *Fusarium* Clade in the *Fusarium solani* species complex, which includes a number of phytopathogens as well as opportunistic pathogens of mammals (O'Donnell et al. 2008, Kasson et al. 2013, O'Donnell et al. 2015). The PSHB (*Euwallacea* sp. #1 in O'Donnell et al. 2015) harbors one of the causative agents of Fusarium dieback, *Fusarium euwallaceae* (Freeman et al. 2013b; AF-2 in O'Donnell et al. 2015). The KSHB (*Euwallacea* sp. #5 in O'Donnell et al. 2015) vectors the other causative agent, *Fusarium kuroshium* (Na et al. 2018; AF-12 in O'Donnell et al., 2015). Both SHB also harbor additional, auxiliary fungal symbionts: PSHB carries *Graphium euwallaceae* and *Paracremonium pembeum* (Lynch et al. 2016), and KSHB carries *Graphium kuroshium* (Na et al. 2018).

Ambrosia beetles form obligate mutualisms with specific fungi, which they actively cultivate and exclusively feed upon. Fungi are transported to new host trees by the beetles in specialized pockets called mycangia, and are deposited within the tree as the beetles bore to create brood galleries for reproduction. The polyphagous and Kuroshio shot hole borers are members of the Xyleborini, a large (~1300 spp.) ambrosia beetle tribe consisting solely of inbreeding haplodiploid species (Normark et al. 1999). Unlike most ambrosia beetles, which colonize dead or dying trees (Raffa et al. 1993), PSHB and KSHB attack living, healthy trees, many of which are susceptible to Fusarium dieback. As the *Fusarium* invades the host tree vascular system, it gradually restricts the flow of water and nutrients (Eskalen et al. 2012). Paired with structural damage caused

by beetle gallery formation, this causes branch dieback and, in some cases, tree death (Eskalen et al. 2012, Mendel et al. 2012).

Sibling-mating paired with arrhenotokous haplodiploidy, as in the Xyleborini, typically results in extremely female-biased sex ratios (Kirkendall 1993). Female SHB disperse having already mated with a brother and carrying *Fusarium* propagules in their mycangia to inoculate brood galleries of their own. However, mating pre-dispersal is not a requirement for female SHB since laying an unfertilized egg will produce a haploid son, which she can then mate with to produce diploid daughters (Cooperband et al. 2016). Combined, these ecological strategies enable SHB to rapidly colonize new areas (Kirkendall and Jordal 2006), and the habit of culturing and feeding on fungi rather than directly on plant material allows them to occupy a wide range of hosts (Jordal 2000).

Although symptoms of Fusarium dieback were recognized much later, the PSHB was first reported in Southern California in 2003 (Rabaglia et al. 2006). Reports of the KSHB in San Diego County began more recently in 2012 (Eskalen et al. 2012). Since their respective invasions, the PSHB and KSHB together have established in five counties in California and are also found in adjacent areas of Mexico (García-Avila et al. 2016; for current distributions in California, see https://ucanr.edu/sites/pshb/Map/). The heart of the PSHB infestation spans Los Angeles and Orange Counties, although they have ranged into neighboring counties as well. KSHB are mostly restricted to San Diego County and northern Mexico, but several specimens have been collected in other California counties farther north (Santa Barbara and San Luis Obispo Counties).

Over fifty tree species common to Southern California are susceptible to Fusarium dieback, including a variety of urban, riparian, and agricultural hosts (Eskalen et al. 2013, Boland 2016, Cooperband et al. 2016). The most notable agricultural host is avocado, which has been threatened by the presence of KSHB in San Diego County and PSHB in Ventura County. California produces 90 percent of domestic avocados, about 70 percent of which are grown in these two counties (40 percent in San Diego, 30 percent in Ventura; California Avocado Commission 2017). In the 2015-2016 season, avocados comprised a $412 million industry in California (California Avocado Commission 2017), the third highest crop value in the history of California avocado production. Since the appearance of Fusarium dieback, the avocado industries of Israel and California have faced losses from damage (Mendel et al. 2012, Freeman et al. 2013b) and although avocado mortality is not as high as originally anticipated (Coleman et al. 2019), the SHB and phytopathogenic *Fusarium* species continue to pose a threat. The beetle-fungus complex has also caused substantial losses in urban environments, where forced removal of thousands of infested landscape trees has cost millions of dollars over the past few years (University of California 2015). Additionally, the beetle-fungus complex is invading natural habitats and threatening native plant species. Over a period of six months, disturbance from KSHB resulted in mortality of the majority of native willows in the Tijuana River Valley in San Diego County (Boland 2016). Willows were the dominant tree species in this riparian habitat that supports numerous plant and animal species, some of which are endangered (Boland 2016). The spread of SHB and their

phytopathogenic fungi therefore have the potential to cause tremendous economic and environmental losses in urban, agricultural, and natural habitats.

Previously, there has been no reliable method of trapping SHB to monitor their distribution and spread. Until recently, the only means of confirming their presence in an area was to find specimens randomly in unbaited traps. Here we present the results of field experiments spanning two years, in which we discover and optimize an effective lure for the polyphagous and Kuroshio SHB: the semiochemical quercivorol. We also report other methods of increasing SHB trap catch through trap modifications, as well as the effects of other potential lures. Finally, we test the effects of the bark beetle deterrent verbenone and another putative deterrent, piperitone, on SHB to determine if and to what extent we can repel them in the field.

MATERIALS AND METHODS

Quercivorol. In a field study to screen various semiochemicals for attraction, Synergy Semiochemicals Corp. (Burnaby, BC, Canada) provided a quercivorol lure (Batch #3250) paired with an ultrahigh release (UHR) bag loaded with ethanol. Together they were found to attract the TSHB in Florida (Carrillo et al. 2015). Quercivorol has also recently been used to capture PSHB in Israel (Byers et al. 2017). We used this lure in an attempt to attract PSHB and KSHB in California.

Quercivorol was first identified from volatiles found in the boring frass of the oak ambrosia beetle *Platypus quercivorus* (Tokoro et al. 2007), for which it has been identified as an aggregation pheromone (Kashiwagi et al. 2006). It has also been found in

odors from artificial media made with boxelder sawdust and infested with the PSHB symbiont, *F. euwallaceae* (Cooperband and Cossé, pers. comm.). Quercivorol ((1S,4R)-*p*-menth-2-en-1-ol) has two chiral centers (Kashiwagi et al. 2006, Tokoro et al. 2007) and four possible enantiomers. SHB may show varying levels of attraction to these different structural isomers, as has been seen in other scolytines (Byers 1989, Byers et al. 2017). We therefore tested different blends of these enantiomers to determine which may be most useful as a lure.

Experimental Design. Experiments were performed in avocado groves in two locations in Southern California: La Habra Heights, Los Angeles County (33°57'33"N, 117°58'10"W) and Escondido, San Diego County (33°08'53"N, 117°01'19"W). Due to their distinct geographical ranges at the time of study, experiments performed in La Habra Heights targeted PSHB, while experiments performed in Escondido targeted KSHB. Experiments were performed sequentially between the summers of 2014 and 2016.

Black 12-funnel Lindgren traps were used for all experiments unless otherwise stated and were hung from vertical metal poles 2.5 meters in height. Poles were bent to a right angle at the top, and traps were secured to the end of the pole so that they hung freely. To prevent poles from being top heavy, 1-meter strips of rebar were hammered into the ground first, and the poles were placed over the rebar to secure them. Traps were spaced roughly 20 meters apart and arranged into randomized complete blocks to control for field location. Whenever trap contents were collected, lures were rotated throughout the block to avoid effects of location bias over the course of the experiment. Lures were

attached to the second lowest funnel of Lindgren traps. Cups were half-filled with propylene glycol antifreeze to collect, euthanize, and preserve specimens (Allison and Redak 2017), which were collected weekly or twice weekly for analysis.

Experiment 1: Shot Hole Borer Attraction to Fungal Odors. Previous studies have shown certain ambrosia beetles to be attracted to the scent of their fungal symbionts (Hulcr et al. 2011, Kuhns et al. 2014). Two novel lures were tested for PSHB attraction: 1) a mixture of their symbiotic fungi *F. euwallaceae* and *G. euwallaceae*, grown on a sawdust-based artificial diet (modified from Peer and Taborsky 2004); and 2) a chemical lure consisting of a quercivorol bubble cap containing 60% *cis*/40% *trans p*-menthenols (Batch #3250, Synergy Semiochemicals) plus a UHR ethanol lure. The artificial diet was prepared with sawdust from box elder, a reproductive host of SHB. 25 ml of autoclaved diet was poured into a 50 ml plastic Falcon tube and allowed to solidify. Separate, equally concentrated spore suspensions of *F. euwallaceae* and *G. euwallaceae* were prepared by the Eskalen lab in the Department of Plant Pathology at the University of California, Riverside, and then combined. 2 ml of the resulting mixture was used to inoculate diet tubes, which were incubated at room temperature (~24°C) for one week before use in Experiment 1. This allowed the fungi enough time to grow over the surface of the diet. The entire fungal-diet mass was removed from each tube in the field and attached to traps using a mesh pocket, to allow fungal scents to escape. Uninoculated diet tubes were prepared and used as a control for SHB attraction to host volatiles in the sawdust. Blank traps served as a negative control. This experiment took place in La Habra Heights for four weeks from Aug-Sep 2014 (N = 28, seven replicates of four

treatments). Trap contents were collected weekly. Because the exposed artificial diet plugs dry out in the field, fresh inoculated and uninoculated diet tubes were prepared weekly to replace old plugs.

Experiment 2: Efficacy of Lindgren Funnel Traps and Purple Sticky Traps. A study was performed to determine if sticky purple prism traps, used for trapping emerald ash borer (Francese et al. 2005), captured more shot hole borers than Lindgren funnel traps. This experiment was performed in tandem with Experiment 1 in La Habra Heights, from Aug-Sep 2014. In this experiment, there were two trap treatments: black 12-funnel Lindgren traps and sticky purple prism traps; as well as two lure treatments: a UHR ethanol lure, and a UHR ethanol lure paired with a quercivorol lure (Batch #3250). The experimental treatments were thus: Lindgren and ethanol, Lindgren and ethanol + quercivorol, prism and ethanol, and prism and ethanol + quercivorol (N = 28, seven replicates of four treatments). Prism traps were replaced weekly, and Lindgren trap contents were collected weekly for analysis.

Experiment 3: Effect of Ethanol on Shot Hole Borer Attraction to Quercivorol. After noting in Experiment 2 that PSHB were not attracted to ethanol lures alone (Fig. 1.2), a study was done to determine if the compound quercivorol performs better alone or if paired with the UHR ethanol lure. Experimental traps were baited with a quercivorol bubble cap (Batch #3250), or with a quercivorol bubble cap and UHR ethanol lure. Blank traps served as a control. This experiment was performed in La Habra Heights for six weeks from Sep-Oct 2014 (N = 45, 15 replicates of three treatments). Trap contents were collected weekly for analysis.

Experiment 4: Effect of α-Phellandrene and β-Phellandrene on Shot Hole Borer Attraction to Quercivorol. We tested for a potential synergistic effect of quercivorol paired with two chemical precursors to quercivorol: α-phellandrene and β-phellandrene. There were two experimental treatments: α-phellandrene paired with quercivorol, and β-phellandrene paired with quercivorol. Traps baited with quercivorol alone served as a positive control. This experiment was performed in La Habra Heights for three weeks in November 2014 (N = 30, 10 replicates of three treatments). Trap contents were collected weekly for analysis.

Experiment 5: Shot Hole Borer Attraction to Three Different Quercivorol Blends. Synergy Semiochemicals Corp. provided lures containing two additional ratios of quercivorol and its stereoisomers (*trans p*-menthenols) for us to test against the original (Batch #3250). The lure contents differed in ratios of different quercivorol enantiomers. Batch #3250 contained 60% *cis*/40% *trans p*-menthenols (load = 280 mg; release rate = 6 mg/day); Batch #3039 contained 26.7% *cis*/53.3% *trans p*-menthenols, 20% piperitols (load = 290 mg; release rate = 6.5 mg/day); and Batch #3355 contained 11% *cis*/87% *trans p*-menthenols (load = 280 mg; release rate = 7.9 mg/day) (D. Wakarchuk, Synergy Semiochemicals, pers. comm., 2016). This experiment was performed in October 2014 in Escondido for three weeks (N = 30, 10 replicates of three treatments). Trap contents were collected weekly.

Experiment 6: Shot Hole Borer Attraction to Two Additional Quercivorol Blends. Two additional lures, Batch #3361 and Batch #3362, were provided by Synergy Semiochemicals Corp. for comparison to Batch #3250. Batch #3361 contained 85%

cis/15% *trans*-*p*-menthenols (load = 280 mg; release rate = 3 mg/day). Batch #3362 contained 57% *cis*/38% *trans*-*p*-menthenols, 5% piperitols (load = 280 mg; release rate = 3 mg/day) (D. Wakarchuk, 2016, personal communication, Synergy Semiochemicals). This experiment was performed in Escondido in March 2015 for two weeks (N = 42, 14 replicates of three treatments). Trap contents were collected twice weekly.

Experiment 7: Comparison of Two Commercially Available Quercivorol Lures. ChemTica Internacional (Santo Domingo, Costa Rica) provided quercivorol lures labeled as P548 (68% *cis*/32% *trans*-*p*-menthenols, load = 200 mg; A. Cossé, 2017, personal communication). We tested these against Synergy's Batch #3361 lure, to see if there was any difference in their attractiveness to SHB. A blank trap served as a control. This experiment took place in Escondido for two weeks from May to June 2015 (N = 30, 10 replicates of three treatments). Trap contents were collected twice weekly.

Experiment 8: Effect of Trap Cup Contents on Shot Hole Borer Capture. Ease of purchase warranted a switch to ethanol-based antifreeze for our field experiments. However, due to hot daytime temperatures and dry conditions in the field, evaporation of ethanol-based antifreeze used in the trap cups resulted in poor morphological and molecular insect preservation. A solution containing dimethyl sulfoxide, EDTA, and saturated NaCl, abbreviated DESS, was previously described for high-temperature preservation of DNA in a variety of animals (Yoder et al. 2006). An experiment was performed to see if DESS solution would affect the number of SHB collected from traps, in order to consider its utility as a preservation agent in the field. The trap cup treatments consisted of ethanol-based antifreeze, DESS solution, or an

empty (dry) cup. DESS solution was prepared at the University of California, Riverside and transported to the field as a liquid. Trap cups were filled halfway for both the antifreeze and DESS treatments. Two moistened, crumpled Kimwipes were placed in dry cups to dissuade captured insects from flying away. A P548 lure was used for all treatments to attract SHB. This experiment was performed in July 2015 in Escondido for two weeks (N = 30, 10 replicates of three treatments). Trap contents were collected twice weekly.

Experiment 9: Effect of Funnel Diameter and Trap Cup Contents on Shot Hole Borer Capture. Due to concerns that live beetles could escape the Lindgren trap cups through the hole of the lowest funnel (6 cm diameter), an experiment was performed to determine if the size of the funnel hole had an effect on the number of SHB collected. In "small" funnel treatments, a plastic funnel with a smaller hole (~3 cm diameter) was glued to the rim of the trap cup to reduce the diameter through which trapped SHB could escape. The effect of trap cup collection substrate was also tested. The treatments were as follows: (1) Lindgren funnel traps with no alterations, here called "large" funnel traps, with dry cups; (2) large funnel traps with cups containing DESS solution; and (3) "small" funnel traps with dry cups. A P548 lure was used for all treatments to attract SHB, and crumpled, moistened Kimwipes were placed inside of dry cups. This experiment was performed in Escondido in July 2015 for two weeks (N = 30, 10 replicates of three treatments). Trap contents were collected twice weekly.

Experiment 10: Effect of Quercivorol Concentration on Shot Hole Borer Attraction. The concentration of a lure has been shown in some systems to determine the

level of attractiveness to a target insect, ranging from attraction to repulsion (Erbilgin et al. 2003, Kovanci et al. 2006, Witzgall et al. 2008). We sought to determine whether the concentration of P548 had an effect on level of SHB attraction. In this experiment, one, two, or six identical P548 lures were attached to a trap to determine the attractiveness of different P548 concentrations to SHB. This experiment was performed in Escondido for six weeks between July and September 2015 (N = 30, 10 replicates of three treatments). Trap contents were collected twice weekly.

Experiment 11: Effect of Quercivorol Release Rate on Shot Hole Borer Attraction. Three P548 lures with varying release rates as described by the company, ChemTica Internacional, were tested. All lures had the same chemical composition and load (200 mg). "P548 A" had the full release rate of 6 mg/day; "P548 B" had a 50% release rate from that of P548 A; and "P548 C" had a 25% release rate from that of P548 A (C. Oehlschlager, 2016, personal communication, ChemTica Internacional). This experiment took place in Escondido for four weeks between September and October 2015 (N = 30, 10 replicates of three treatments). Trap contents were collected twice weekly.

Experiment 12: Effect of the Repellent Verbenone on Shot Hole Borer Attraction to Quercivorol. To see if we could repel SHB in the field, ChemTica Internacional (Santo Domingo, Costa Rica) provided pouches of Beetleblock Verbenone. Verbenone has been used in the past to successfully deter economically important bark beetles in the genera *Ips* and *Dendroctonus* (Borden et al. 1991, Fettig et al. 2009), and has more recently been utilized for ambrosia beetle pests (Burbano et al. 2012, Jaramillo et al. 2013, Hughes et al. 2017). We tested the effect of verbenone on SHB by pairing the

verbenone pouch with a quercivorol lure (Batch #3361; Synergy Semiochemicals, Burnaby, BC, Canada), to determine if the repellent offset the attractiveness of quercivorol. For a positive control we used a Batch #3361 lure alone, and for a negative control a blank trap was used. This experiment was performed in La Habra Heights for three weeks between Oct-Nov 2015 (N = 30, 10 replicates of three treatments). Trap contents were collected weekly.

Experiment 13: Effect of the Repellent Piperitone on Shot Hole Borer Attraction to Quercivorol. We tested the effects of verbenone against another repellent, piperitone (Synergy Semiochemicals) to determine which deters SHB more effectively. Piperitone was tested because it is the ketone form of the attractant quercivorol, similar to verbenone being the ketone form of the bark beetle attractant verbenol. This experiment was the first to test piperitone as a repellent against ambrosia beetles. Similar to Experiment 12, both repellents were paired with a quercivorol lure (Batch #3361; Synergy Semiochemicals) and were tested against a Batch #3361 lure as a positive control. This experiment was performed in La Habra Heights and lasted for six weeks between August and September 2016 (N = 30, 10 replicates of three treatments). Trap contents were collected weekly.

Statistical Analysis. All statistical analyses were performed using the R free software v3.2.1 (R Core Team 2017). For all experiments, data was collected in the form of counts, and were found in all cases to be Poisson overdispersed (Pearson dispersion statistic >1.0). Data were analyzed using a negative binomial regression, using the *glm.nb* function in the 'MASS' package (Venables and Ripley 2002) in R to employ a

generalized linear model under the assumptions of a negative binomial distribution. The number of SHB captured was modeled by the effects of treatment, date, and block. Multiple comparisons were performed using Tukey contrasts of least-squares means, using the 'lsmeans' package (Lenth 2016). To account for outliers, analyses were performed both before and after removing outliers from the data set. Any significant effects of outliers are discussed. Results are reported as raw count data. Box plots for each experiment show sample minimum and maximum (horizontal lines at the bottom and top of each plot, respectively) as well as sample median (heavy line inside of box). Upper and lower quartiles are represented by the upper and lower limits of each box, respectively. Data points that fall outside of the quartile ranges are denoted as open circles. Letters indicate significance bins at $\alpha = 0.05$. Summary statistics and Pearson's dispersion statistic are also reported for each experiment (Table 1.1).

RESULTS

Experiment 1: Shot Hole Borer Attraction to Fungal Odors. We found that the Batch #3250 quercivorol + UHR ethanol lure attracted significantly more SHB ($\chi^2 = 665.16$; df = 3; $P < 0.001$; Fig 1.1) than either the inoculated or uninoculated diet plug, neither of which were significantly different from our blank control trap ($P = 0.876$ and 0.729, respectively).

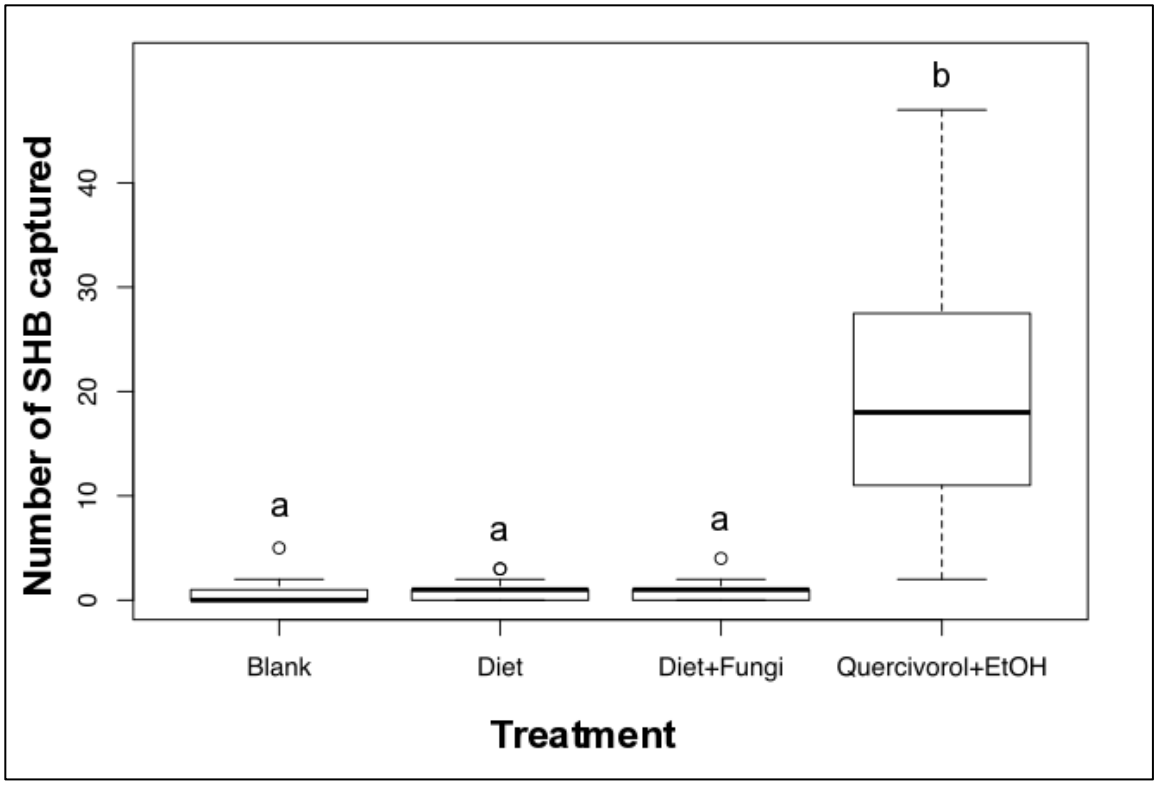


Fig. 1.1 Shot Hole Borer Attraction to Fungal Odors. The paired Batch #3250 quercivorol + UHR ethanol lure attracted significantly more SHB than either the inoculated or uninoculated diet tubes ($P < 0.001$), neither of which was significantly different from the blank control trap ($P = 0.876$ and 0.729, respectively).

Experiment 2: Efficacy of Lindgren Funnel Traps and Purple Sticky Traps. Purple sticky prism traps captured significantly more SHB than 12-funnel black Lindgren traps, regardless of lure treatment ($\chi^2 = 203.49$; df = 3; $P < 0.001$; Fig. 1.2). For both trap types, significantly more SHB were captured using a combination lure of ethanol + quercivorol than using ethanol alone.

Experiment 3: Effect of Ethanol on Shot Hole Borer Attraction to Quercivorol. We found that the Batch #3250 quercivorol lure by itself attracted significantly more SHB than when the lure is paired with a UHR ethanol lure ($\chi^2 = 1221.03$; df = 2; $P < 0.001$; Fig 1.3). Both treatments resulted in significantly higher SHB capture than blank control traps (both $P < 0.001$).

Experiment 4: Effect of α-Phellandrene and β-Phellandrene on Shot Hole Borer Attraction to Quercivorol. We found that, rather than being synergistic, the addition of α- or β-phellandrene greatly reduced SHB attraction to quercivorol ($\chi^2 = 86.49$; df = 2; $P < 0.001$; Fig. 1.4). There was no difference between α-phellandrene and β-phellandrene in this respect ($P = 0.214$).

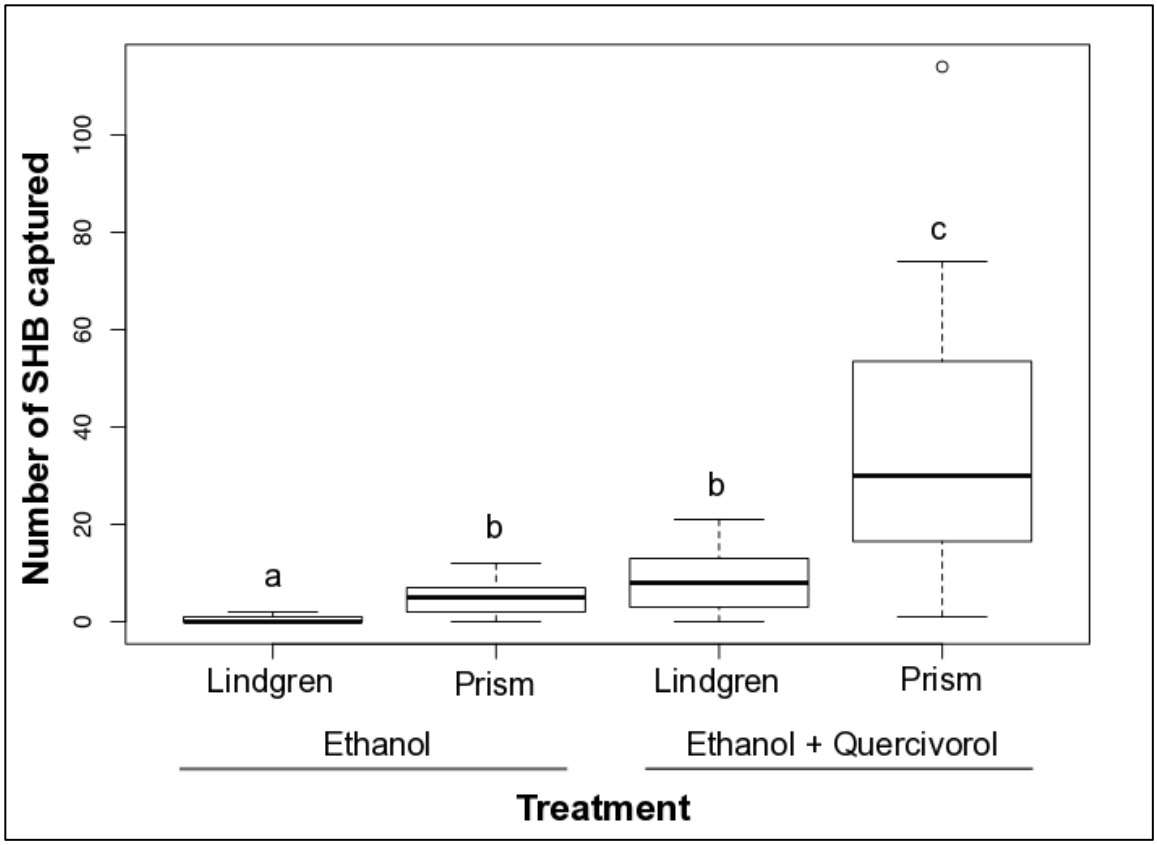


Fig. 1.2 Efficacy of Lindgren Funnel Traps and Purple Sticky Traps. Purple prism traps were more efficient at capturing SHB than Lindgren traps, regardless of lure treatment ($P < 0.001$ for both lure treatments). The ethanol + quercivorol lure treatment captured more SHB than the ethanol treatment ($P < 0.001$ for both trap treatments).

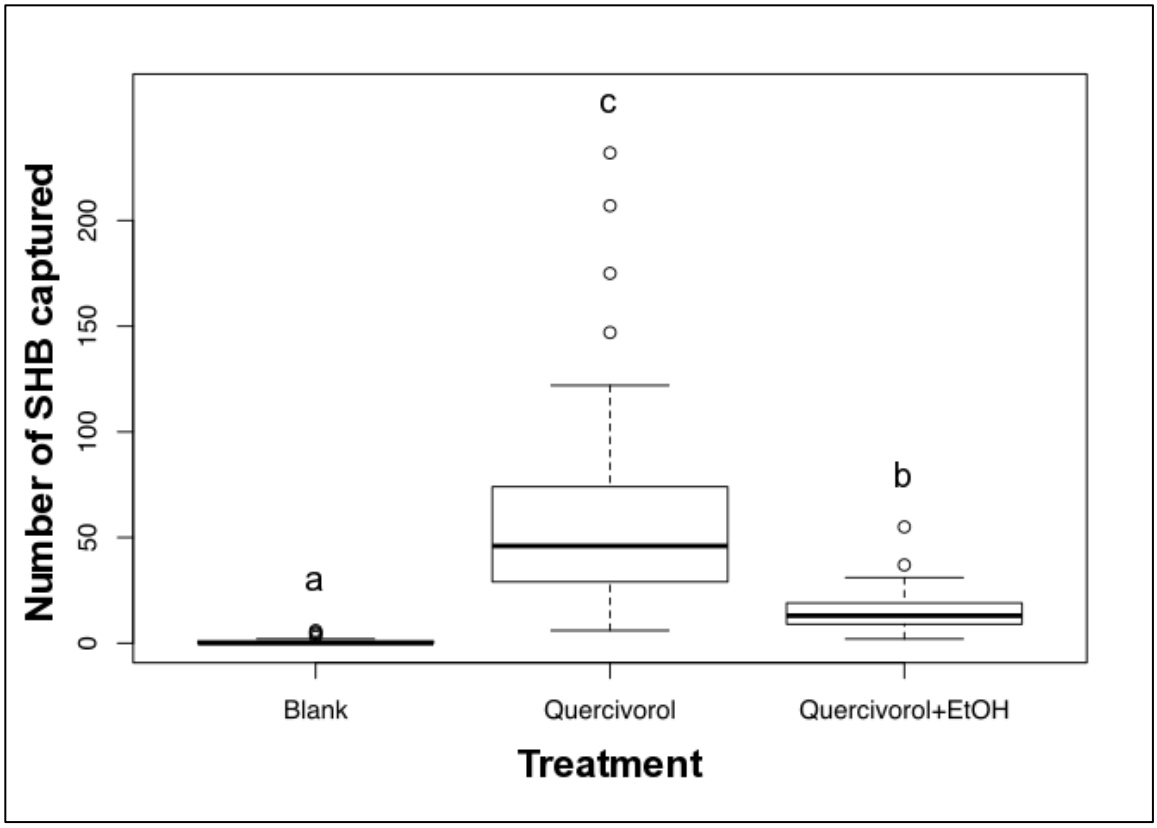


Fig. 1.3 Effect of Ethanol on Shot Hole Borer Attraction to Quercivorol. The Batch #3250 quercivorol lure by itself attracted significantly more SHB than when paired with a UHR ethanol lure ($P < 0.001$). Both treatments attracted significantly more SHB than the blank control trap ($P < 0.001$).

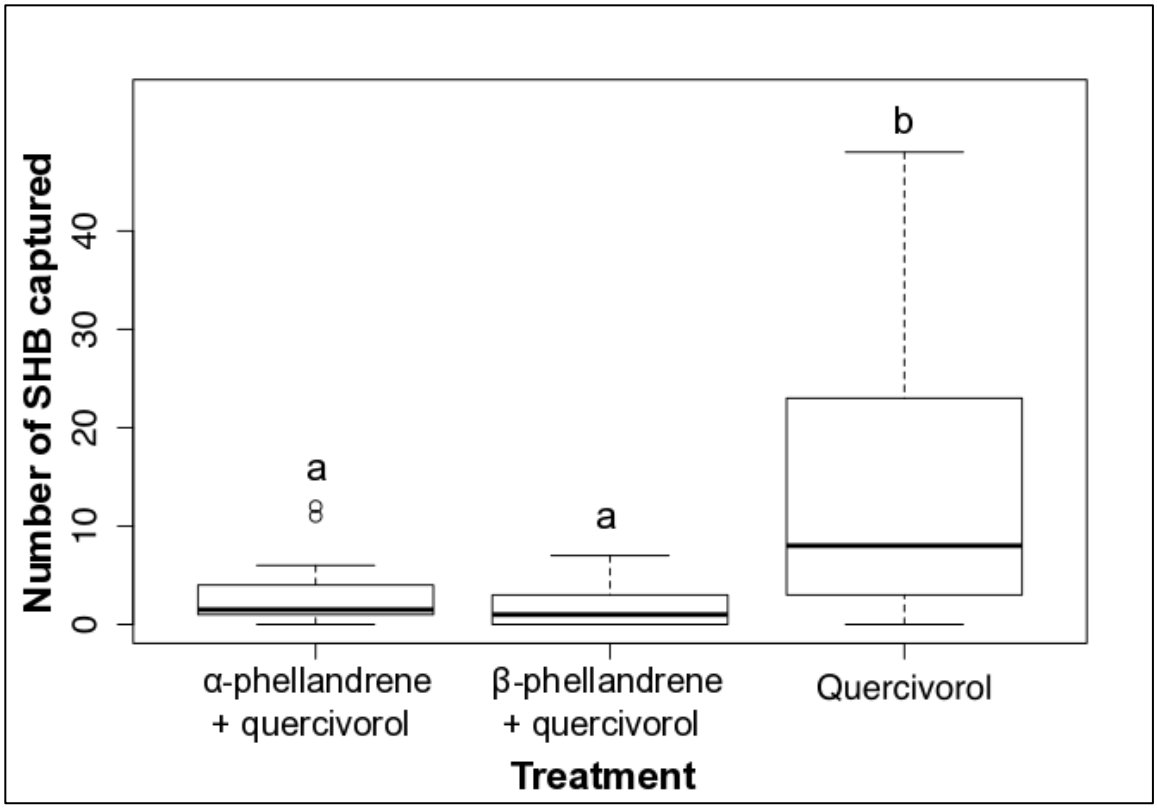


Fig. 1.4 Effect of α-Phellandrene and β-Phellandrene on Shot Hole Borer Attraction to Quercivorol. SHB attraction to quercivorol was significantly reduced with the addition of either α-phellandrene or β-phellandrene (both $P < 0.001$). There was no difference in number of SHB captured between the two phellandrene treatments ($P = 0.214$).

Experiments 5 & 6: Shot Hole Borer Attraction to Different Quercivorol Blends. We found that the Batch #3250 quercivorol lure attracted significantly more SHB than Batch #3039 ($\chi^2 = 134.66$; df = 2; $P < 0.001$) and Batch #3355 ($P < 0.001$; Fig 1.5a). Batch #3039 attracted significantly more SHB than Batch #3355 ($P < 0.001$). Additionally, we found no significant difference between Batch #3361 and Batch #3250 ($\chi^2 = 25.97$; df = 2; $P = 0.427$; Fig. 1.5b). Both of these batches attracted significantly more SHB than Batch #3362 ($P < 0.001$).

Experiment 7: Comparison of Two Commercially Available Quercivorol Lures. We found no significant difference between the number of SHB attracted to the P548 and Batch #3361 quercivorol lures ($\chi^2 = 47.33$; df = 2; $P = 0.311$; Fig. 1.6). Both of these treatments attracted significantly more SHB than the blank control traps ($P < 0.001$).

Experiments 8 & 9: Effect of Funnel Diameter and Trap Cup Contents on Shot Hole Borer Capture. We found significantly more SHB in cups containing DESS solution than either in cups with antifreeze ($\chi^2 = 19.53$; df = 2; $P = 0.010$) or dry cups with Kimwipes ($P < 0.001$; Fig. 1.7a). There was no significant difference between the number of SHB collected in cups with antifreeze or in dry cups ($P = 0.359$). When dry cups were used, we found that the diameter of funnels had no effect on how many SHB were caught ($\chi^2 = 9.18$; df = 2; $P = 0.999$; Fig. 1.7b). However, significantly more SHB were collected in cups containing DESS than in dry cups of either large or small funnel traps (both $P = 0.025$).

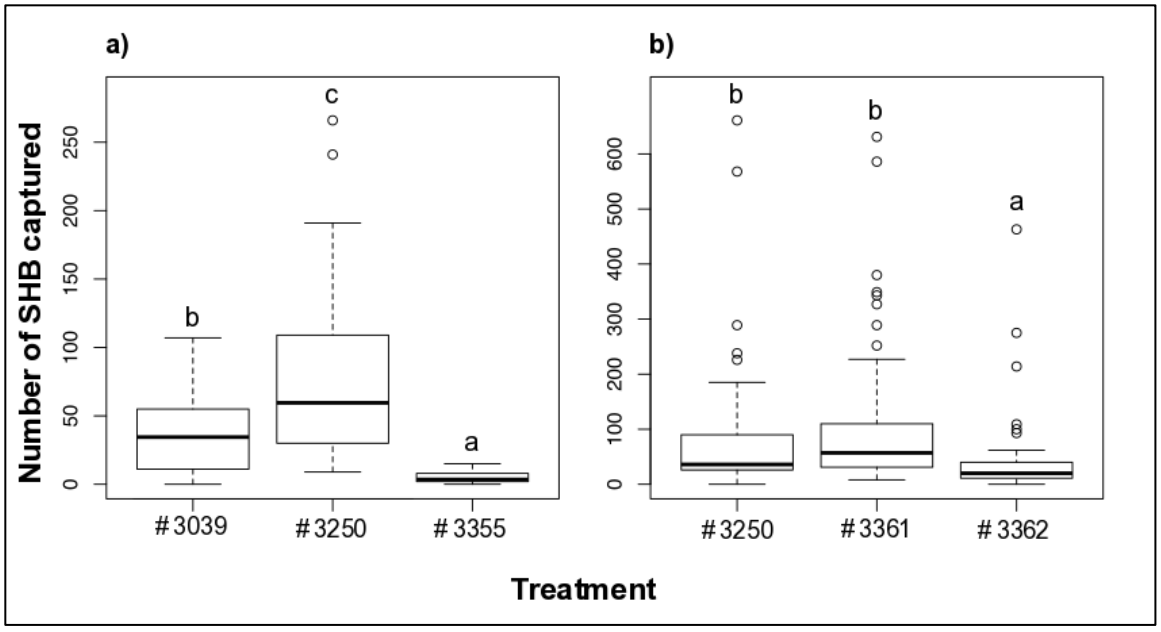


Fig. 1.5 Shot Hole Borer Attraction to Different Quercivorol Blends. (a) Significantly more SHB were attracted to Batch #3250 than to either Batch #3039 ($P < 0.001$) and Batch #3355 ($P < 0.001$). Batch #3039 attracted significantly more SHB than Batch #3355 ($P < 0.001$). **(b)** There was no significant difference in number of SHB attracted to Batch #3250 and Batch #3361 ($P = 0.427$), both of which attracted significantly more SHB than Batch #3362 ($P < 0.001$).

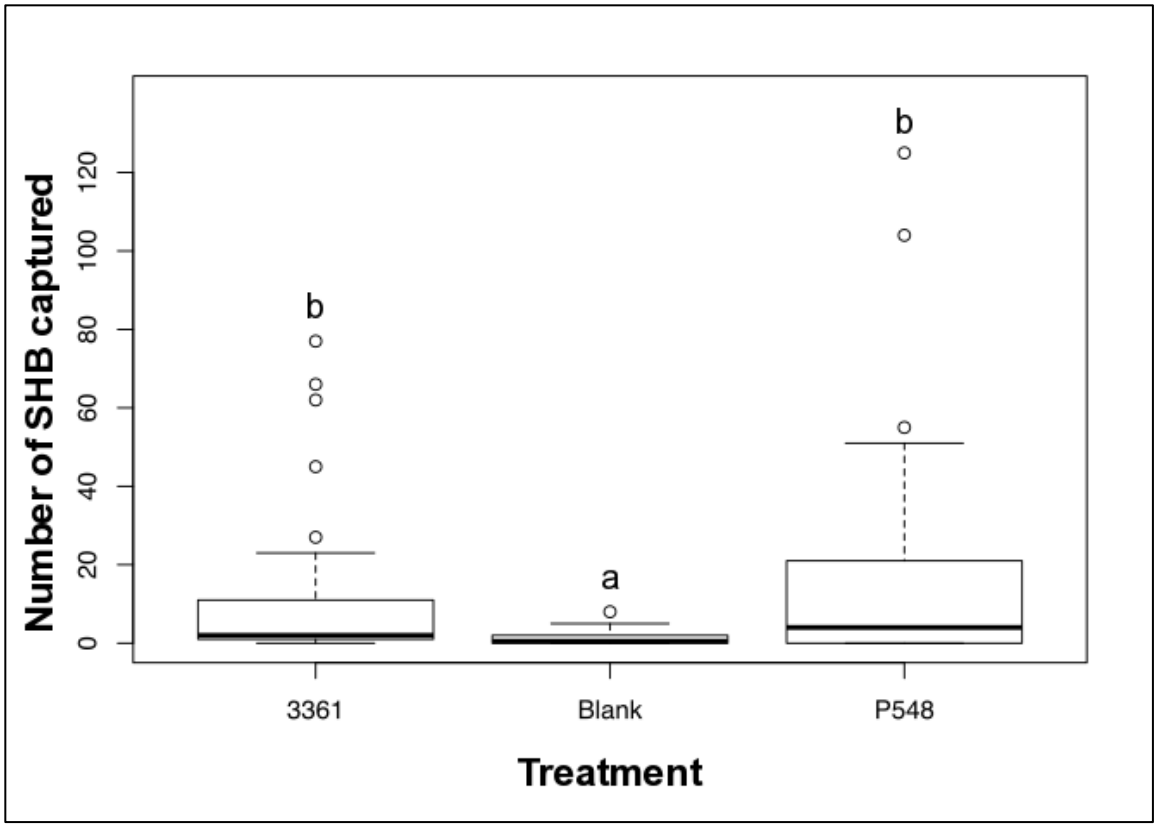


Fig. 1.6 Comparison of Two Commercially Available Quercivorol Lures. There was no significant difference in the number of SHB collected from traps baited with Batch #3361 (Synergy Semiochemicals) or with P548 (ChemTica Internacional; P = 0.311).

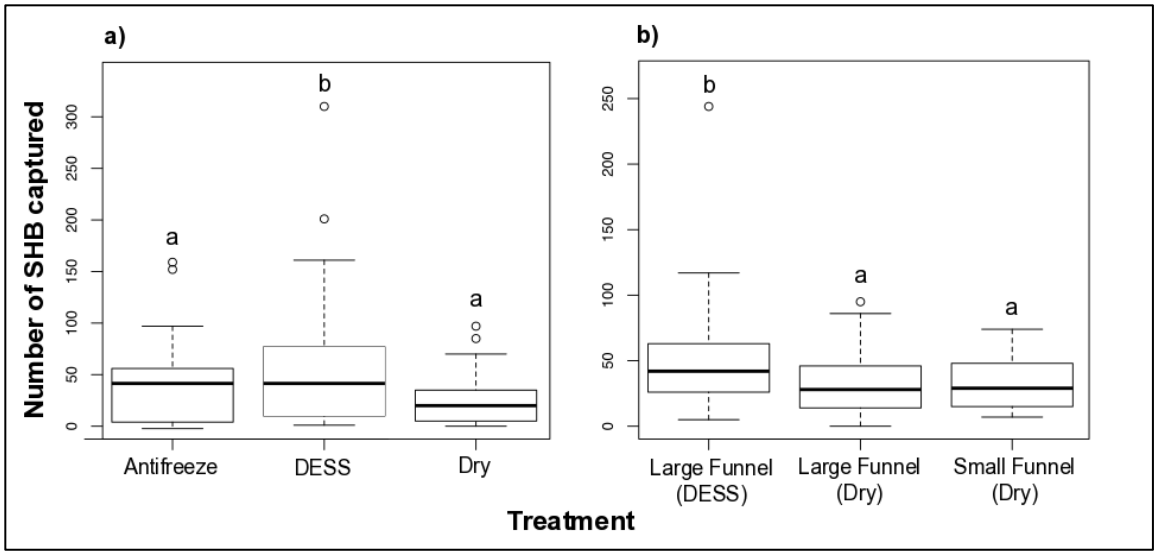


Fig. 1.7 Effect of Funnel Diameter and Trap Cup Contents on Shot Hole Borer Capture. (a) Significantly more SHB were collected from trap cups containing DESS solution than either cups with antifreeze ($P = 0.010$) or dry cups ($P < 0.001$). **(b)** Size of funnel diameter had no effect on number of SHB captured ($P = 0.999$). Significantly more SHB were collected from cups contained DESS solution than dry cups of either large or small funnel traps (both $P = 0.025$).

Experiments 10 & 11: Effect of Quercivorol Concentration and Release Rate on Shot Hole Borer Attraction. Significantly more SHB were attracted to a single P548 lure than to the six-lure treatment ($\chi^2 = 23.14$; df = 2; $P < 0.001$; Fig. 1.8a). We found no significant difference in the number of SHB captured with the single lure compared to the two-lure treatment ($P = 0.259$). We found no difference in the number of SHB attracted to P548 A, P548 B, and P548 C with different release rates ($\chi^2 = 3.06$; df = 2; treatment effect $P = 0.315$; Fig. 1.8b).

Experiments 12 & 13: Effect of the Repellents Verbenone and Piperitone on Shot Hole Borer Attraction to Quercivorol. As expected, the Batch #3361 quercivorol lure as a positive control attracted a significant number of SHB weekly ($\chi^2 = 396.20$; df = 2; $P < 0.001$; Fig. 1.9a). When paired with a Batch #3361 quercivorol lure, verbenone significantly reduced the number of SHB attracted to the quercivorol lure ($P < 0.001$), although it still attracted significantly more SHB than the blank control trap ($P < 0.001$). We found that significantly fewer SHB were collected from traps with piperitone than traps with verbenone ($\chi^2 = 306.47$; df = 2; $P < 0.001$; Fig. 1.9b). Both repellents significantly reduced the number of SHB attracted to the Batch #3361 quercivorol lure ($P < 0.001$).

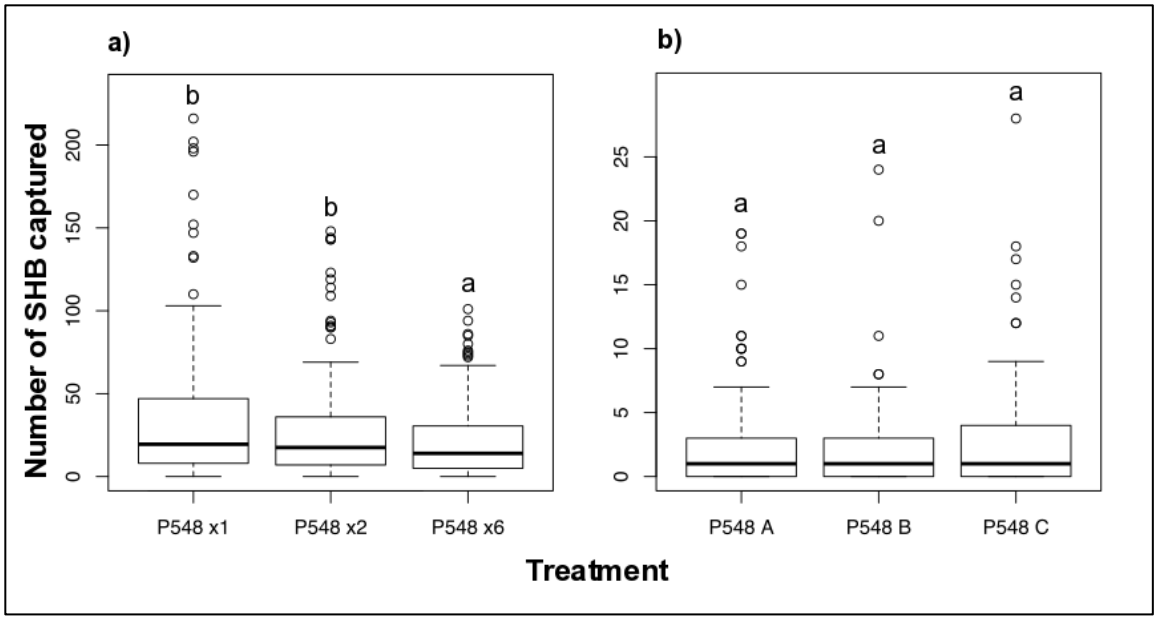


Fig. 1.8 Effect of Quercivorol Concentration and Release Rate on Shot Hole Borer Attraction. (a) Significantly more SHB were attracted to the one- and two-lure treatments than the six-lure treatment ($P < 0.001$). There was no significant difference between the one- and two-lure treatments ($P = 0.259$). **(b)** Release rate had no significant effect on number of SHB captured (treatment $P = 0.315$).

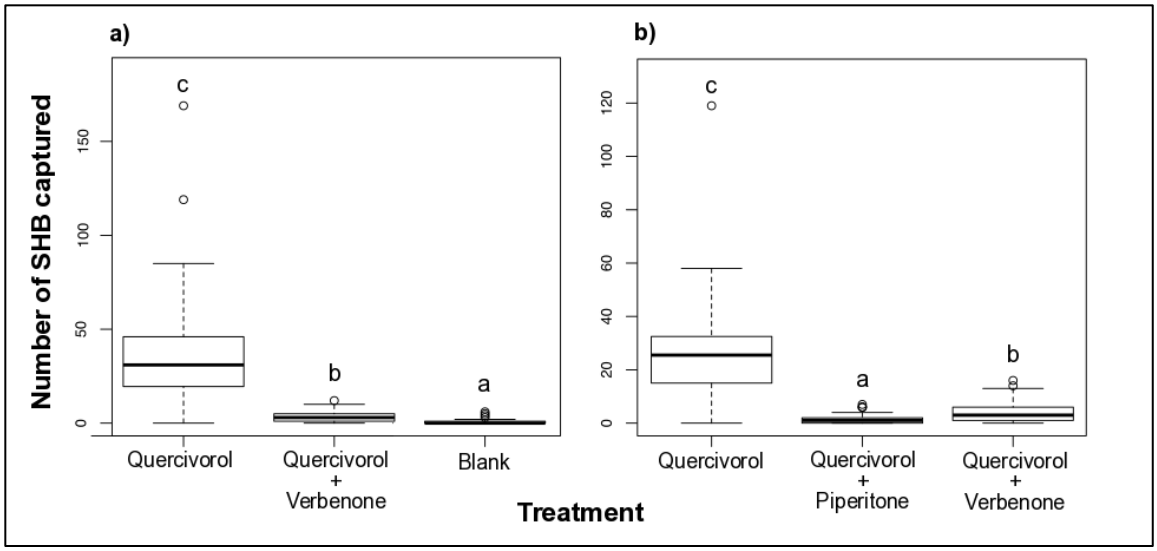


Fig. 1.9 Effect of the Repellents Verbenone and Piperitone on Shot Hole Borer Attraction to Quercivorol. (a) Verbenone significantly reduced the number of SHB attracted to quercivorol ($P < 0.001$), although this paired lure still attracted more SHB than the blank control trap ($P < 0.001$). **(b)** When both were paired with quercivorol, significantly fewer SHB were collected from traps with piperitone than traps with verbenone ($P < 0.001$). Both repellents significantly reduced SHB attraction compared to the quercivorol lure alone ($P < 0.001$).

DISCUSSION

Our experiments revealed that lures containing quercivorol are highly effective for the capture of PSHB and KSHB. Although quercivorol has been found in odors from sawdust-based artificial diet infested with *Fusarium euwallaceae* (Cooperband & Cossé, personal communication), we found that SHB were not attracted in the field to diet plugs inoculated with symbiotic fungi in Experiment 1 (Fig. 1.1). Fungal volatiles from these lures may have been emitted at concentrations below the threshold of beetle detection under field conditions. We found that removing the UHR ethanol component greatly increased the ability of quercivorol lures to attract SHB (Fig. 1.3), suggesting that, unlike many other bark and ambrosia beetles that are attracted to UHR ethanol (Montgomery and Wargo 1983, Schroeder and Lindelöw 1989, Miller and Rabaglia 2009), PSHB and KSHB have an aversion to UHR ethanol, or that ethanol at that release rate has an antagonistic effect on quercivorol. We have also shown that SHB have an aversion to the repellents verbenone (Fig. 1.9a) and piperitone (Fig. 1.9b), which almost completely offset SHB attraction to quercivorol. We found that piperitone is a more effective deterrent for SHB than verbenone (Fig. 1.9b), making this study the first to demonstrate the potential of piperitone for ambrosia beetle control.

Our experiments show that SHB respond differently to different ratios of quercivorol isomers. Both SHB seem to be most attracted to blends where *cis* quercivorol is the dominant isomer (Fig. 1.5), and we found no significant difference in their attraction to quercivorol lures from Synergy Semiochemicals Corp. and ChemTica Internacional (Fig. 1.6). Additionally, we found that SHB do not respond differently to

quercivorol lures with different release rates (Fig. 1.8b). We did find, however, that SHB show higher attraction to lower concentrations of quercivorol (Fig. 1.8a) than higher concentrations. Lower SHB catch from higher concentration treatments may reflect either a lessened attraction compared to the low concentration treatment, or possibly an aversion to such high concentrations. These findings allow for more cost-effective monitoring, since lures with lower concentrations or release rates are typically less expensive to synthesize and purchase than high concentration, full release rate lures.

Attempts to modify traps to increase SHB catch were somewhat successful. Although alteration of funnels had no effect on the number of SHB being retained (Fig. 1.7b), we found that using DESS solution as a cup substrate resulted in higher numbers of SHB in trap cups than when ethanol-based antifreeze was used (Fig. 1.7a). We cannot rule out the possibility that these results were caused by other factors, but this again suggests that SHB have an aversion to ethanol, and also has implications for the use of DESS solution as a field preservation agent.

Because count data is typically skewed, the data were not transformed and are reported as raw count data. However, the possible effect of outliers cannot be ignored. Removing outliers changed significance of the results in one of our experiments. In Experiment 9, the difference between cups with DESS and dry cups was no longer significant at $\alpha = 0.05$ after removing outliers ($P = 0.074$ and 0.099 for large and small traps, respectively). The effect of date of collection from week to week was also significant in some experiments, which may have influenced the presence of outliers. There are two main explanations for this observation, the first being dosage effects that

gradually diminished lure potency over the course of the experiments. This was a known and uncontrollable factor in our experiments, but one that was unlikely to differentially affect our results since all lures had comparable loads and release rates (except in Experiment 11, where the effect of release rate was tested). The second explanation is temperature, which, in addition to affecting release rate, could have caused an overall increase or decrease in number of flying SHB (i.e., the pool from which SHB could be collected in the field) and therefore likely would have affected all treatments equally. Thus, the effect of date likely did not affect comparisons between treatments.

Because PSHB and KSHB are members of a closely related species complex with similar fungal symbionts, we assume that their responses to the lures and repellents tested are comparable. Synergy Semiochemical and ChemTica quercivorol lures have both been used in the field experiments described here in La Habra Heights and Escondido, and have resulted in sufficient capture of SHB in each location. Both quercivorol lures are also currently used for monitoring purposes across southern California (see https://ucanr.edu/sites/pshb/Map/), and have resulted in capture of both PSHB and KSHB in their respective locations. It is therefore safe to say that both SHB are attracted to quercivorol; however, there may be differences in their responses to other lures or repellents.

Due to various aspects of their ecologies, bark and ambrosia beetles are notoriously difficult to control. Females spend most of their lives protected within host trees, and disperse already mated with their fungal symbionts. Dispersal typically occurs over a short distance in one of two ways: a flight to another suitable host, or to walk to an

unoccupied area of the current host tree. These factors reduce the need for sex or aggregation pheromones in SHB, and indeed none have been discovered. Without the utility of artificially synthesized pheromones or ethanol lures to attract the PSHB and KSHB, the discovery of quercivorol has been a great advance to our knowledge of SHB distribution and spread. Results from our field experiments have greatly optimized SHB trap catch and resulted in an effective monitoring tool for these invasive pests. Monitoring PSHB and KSHB has previously required field surveys of Fusarium dieback symptoms. Surveys of this kind are time-consuming and rely on accurate and complete visual diagnosis by the surveyor. The development of effective lures provides for passive and less subjective monitoring. Quercivorol could also potentially be used to control SHB through an attract-and-kill type strategy: optimization of both lure and trap could help in decreasing overall SHB population numbers in infested areas, limiting opportunities for the beetle to spread. Paired with effective placement of piperitone or other repellents, this could help to protect uninfested areas from SHB attack.

Table 1.1 Minimum, maximum, and mean values of number of shot hole borers captured per trap for each experiment. Standard error of the mean and Pearson's dispersion statistic is also reported.

Experiment #	Min	Max	Mean	SE	Pearson's dispersion statistic
1	0	47	5.42	0.93	1.815765
2	0	114	10.96	1.90	6.72542
3	0	232	24	2.05	8.96037
4	0	48	5.77	0.96	3.598702
5	0	266	41.21	5.72	18.6935
6	2	661	89.27	11.72	75.4172
7	0	125	10.76	2.39	15.7298
8	0	310	44.83	5.52	21.6067
9	0	244	40.41	3.57	23.6081
10	0	216	30.24	1.94	22.3596
11	0	28	2.49	0.24	4.07333
12	0	169	14.26	2.31	7.38229
13	0	119	10.43	1.15	5.24621

Chapter II. Effect of temperature on development and emergence of the invasive ambrosia beetle *Euwallacea kuroshio* (Coleoptera: Scolytinae) in Southern California

ABSTRACT

We performed a series of rearing trials at a range of constant temperatures to experimentally determine the effect of temperature on development and emergence of the Kuroshio shot hole borer *Euwallacea kuroshio*, an invasive ambrosia beetle pest in Southern California. We monitored daily emergence at each temperature to determine the i) number of successful colonies established at each temperature, ii) mean number of days until first offspring emergence, iii) mean number of offspring per colony, and iv) mean number of offspring emerged daily. We found that *E. kuroshio* had the highest total emergence and number of successful colonies when reared at 28°C, and that mean days until first offspring emergence decreased as temperature increased up to 30°C, after which emergence ceased. Emergence was low below 20°C. We found that *E. kuroshio* needs 318 degree-days to complete its development and emerge. We pair our experimental data with publically available weather data to predict the annual number of generations of *E. kuroshio* across its invasive range in California.

INTRODUCTION

Temperature plays a vital role in the development of ectothermic organisms, and dictates the environments suitable for particular species. In insects, studies of temperature effects on development are valuable for the formulation of degree-day (DD) models, which are used to predict emergence and flights of pestiferous insects. To create these models, a pest species is typically reared under a range of constant temperatures and the time required for the development of different life stages is recorded.

The Kuroshio shot hole borer (KSHB) *Euwallacea kuroshio* Gomez & Hulcr (2018) is a pest of at least fifteen tree species in Southern California (Stouthamer et al. 2017, Eskalen 2019a). It is an ambrosia beetle in the subfamily Scolytinae (Coleoptera: Curculionidae), a group of minute wood-borers that occupy the xylem of host trees and form obligate mutualisms with fungi. KSHB is associated with the fungi *Fusarium kuroshium* and *Graphium kuroshium*, which serve as the beetles' sole food source throughout their development (Na et al. 2018). When females disperse to form brood galleries and reproduce, they transport these fungi between host trees inside of paired specialized organs called mycangia. *F. kuroshium* and *G. kuroshium* are both phytopathogens (Na et al. 2018) and, once established, can block host tree vascular transport and restrict water flow. The dual effects of the phytopathogens and boring damage from KSHB cause the plant disease Fusarium dieback (Eskalen et al. 2013).

KSHB is one of four known members of the cryptic *Euwallacea fornicatus* species complex (Stouthamer et al. 2017, Gomez et al. 2018). The current distribution of KSHB in California ranges through San Diego, Orange, and Los Angeles Counties, with

recent establishments farther north in Santa Barbara and San Luis Obispo Counties (Eskalen 2019b). Two other members of this species complex are also invasive to the United States; these are the polyphagous shot hole borer (PSHB) *Euwallacea fornicatus* Eichhoff, which is also established in California, and the tea shot hole borer (TSHB) *Euwallacea perbrevis* Schedl, which is invasive in Florida and Hawaii (O'Donnell et al. 2015, Stouthamer et al. 2017, Smith et al. 2019). All three are capable of spreading Fusarium dieback, and threaten commercial avocado production (Mendel et al. 2012, Carrillo et al. 2016, Lira-Noriega et al. 2018) as well as a growing number of hosts in natural and urban landscapes (Eskalen et al. 2013, Boland 2016, Owens et al. 2018).

Much work has been done on the emergence and flight ecology of the TSHB, which has been a significant pest of tea in Southeast Asia for over a century (Gadd 1941). In their native range, TSHB only emerge and fly between the hours of 9:00-16:00 (10:00-17:00 DST), with about 85% of daily catch occurring between 11:00-14:00 (12:00-15:00 DST; Calnaido 1964, 1965). More recently, this general diurnal time frame was confirmed for TSHB in its invasive range in Florida (Kendra et al. 2017, Owens et al. 2019) and has also held true for PSHB and KSHB in Southern California (pers. obs.). It was also found that TSHB emergence is not necessarily synchronized with flight, which is heavily dependent on temperature and other abiotic factors present outside of the host plant (Calnaido and Thirugnanasuntharan 1966).

Due to their status as pests, the effect of temperature on development rates of various life stages of both PSHB and TSHB have previously been reported (Gadd 1946, Walgama and Zalucki 2007, Umeda and Paine 2018), and it is assumed that all four

species have similar temperature requirements since they are closely related and occupy similar native ranges in Southeast Asia (Stouthamer et al. 2017). However, these prior studies focused on time to development for different beetle life stages, and did not take emergence rate into account. Adult female ambrosia beetles may reside in the gallery for extended periods post-development (Peer and Taborsky 2006), and their emergence cannot be predicted from developmental data alone. We hope to build on previous studies using contemporary empirical emergence data. We synthesize our emergence data with publically available environmental data to predict the annual number of KSHB generations in four locations spanning its invasive range in California.

MATERIALS AND METHODS

Temperature Study. KSHB were reared under a series of constant temperatures: 16, 18, 20, 24, 26, 28, 30, and 32 (°C). A total of thirty-six single-foundress colonies were initiated for each temperature trial. Colonies were propagated in 50 mL centrifuge tubes containing sawdust-based artificial media (modified from Peer and Taborsky 2004, Cooperband et al. 2016) to promote the growth and development of ambrosia beetles and their symbiotic fungi. To make the media, the following dry ingredients were combined in a 1 L autoclavable bottle: 75 g finely-milled avocado sawdust, 20 g agar (BD Difco™, Sparks, MD, USA), 10 g sucrose (Research Products International©, Mount Prospect, IL, USA), 5 g corn starch (Fisher Scientific™, Nazareth, PA, USA), 5 g yeast (Fisher Scientific™, Nazareth, PA, USA), 5 g casein (Spectrum Chemical®, Gardena, CA, USA), 1 g Wesson's salt mixture (MP Biomedicals™, Solon, OH, USA). The following wet

ingredients were then added to the bottle: 2.5 mL wheat germ oil (Viobin®, Monticello, IL, USA), 5 mL 95% ethanol, and 500 mL deionized water. The contents of the flask were thoroughly mixed by shaking, then autoclaved at 121°C for 30 min. Once sterilized, the media was poured into 50 mL Falcon™ tubes (BD Biosciences, Bedford, MA, USA) up to the 20 mL line. Caps were placed loosely on the tubes to allow condensation to evaporate (4-7 days). Once dry, a single hole was made in the media using a sterilized instrument, to facilitate gallery initiation by KSHB. KSHB were originally collected live from Escondido, CA between the years 2014 and 2016, and subsequent generations were reared at the University of California, Riverside Insectary and Quarantine Facility. Each colony tube received a single recently eclosed, mature, mated adult female KSHB (i.e., a foundress). Foundresses were surface sterilized for 5 s in 70% ethanol before being placed in the tube, and were allowed 1-3 days post-introduction to establish a gallery within the media. The tube caps were then removed and replaced with a ring that facilitated attachment of the rearing tube to another 50mL Falcon™ tube, with a modification: a 1 cm diameter hole was bored through the plastic, and then covered with a square 1 cm^2 piece of fine metal mesh (Fig. 2.1). This allowed air to circulate in the tube and prevented condensation buildup. The ring was created with a Bukobot V2 Vanilla 3-D printer (Deezmaker, San Dimas, CA, USA) using acrylonitrile butadiene styrene (ABS) filament (Prototype Supply, Shenzhen, China). The attached tubes were inverted, such that the rearing tube with artificial media was on top and the ventilation tube was on the bottom (Fig. 2.1), and secured in a Styrofoam rack. Inverting the tubes caused emerging adult offspring to fall to the bottom of the tubes

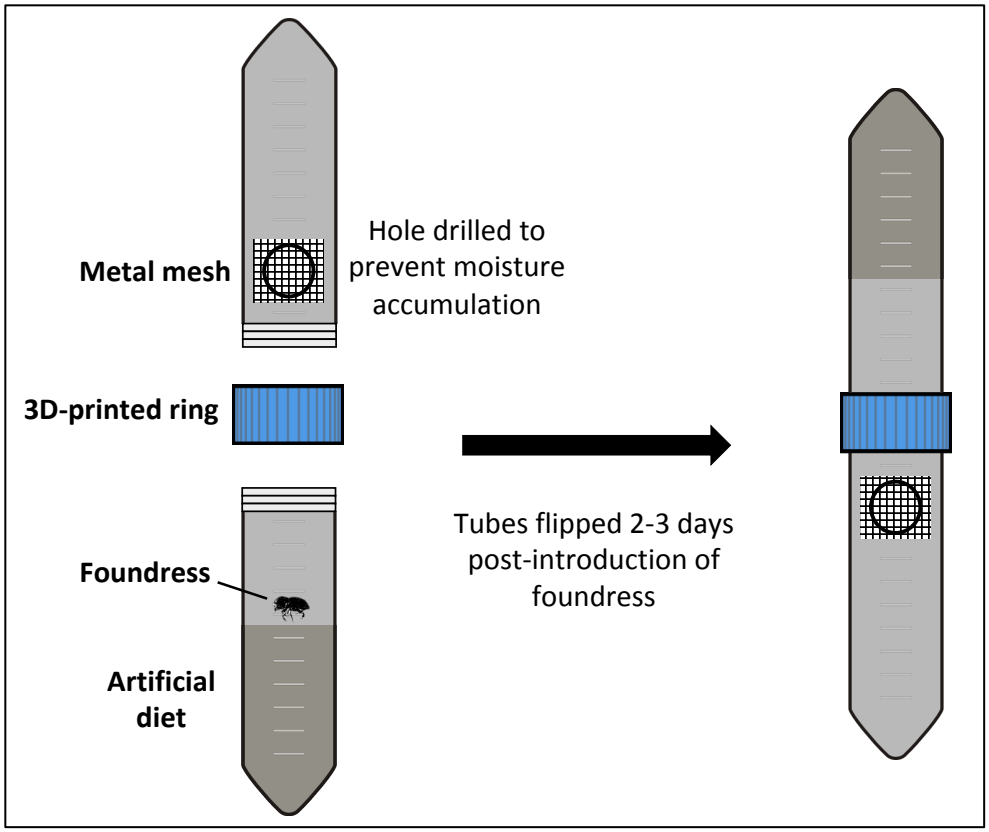


Fig. 2.1 Schematic of rearing tube set-up. Individual tubes were secured in a Styrofoam rack within the rearing chamber.

post-emergence and, importantly, prevented them from burrowing back into the media, allowing for efficient and accurate daily collection.

For each trial, the thirty-six rearing tubes were placed in growth chambers (Model I-30BLL, Percival Scientific, Perry, IA, USA) set at a constant temperature and relative humidity (50 ± 10%). The growth chambers were set to a photoperiod of 0L:24D, to mimic the beetles' natural conditions inside a host tree (Walgama and Zalucki 2007). To capture offspring emergence from at least one complete generation, trial duration at the warmer temperatures (24, 26, 28, 30, and 32°C) lasted from 56-112 days; the coldest

temperature trials (16, 18, and 20°C) were terminated 185 days post-introduction. The number of adult females that emerged was counted daily, and hereafter the term "offspring" refers only to adult female offspring, unless otherwise stated. Daily counts were performed after 17:00 DST, when KSHB no longer emerge. All colony tubes were dissected at the end of the 16, 18, and 32°C trials to determine if offspring were remaining inside instead of emerging at these temperatures.

The number of colony tubes that produced emerging offspring (N; Table 2.1) was recorded for each temperature. The number of days until the first offspring emerged was recorded for each colony tube (Table S2.1). These emergence dates were averaged across all tubes within a temperature trial to give the mean number of days until offspring emergence ("Days until emergence"; Table 2.1). To estimate how temperature affects emerging brood size, the days until emergence was doubled for each temperature trial, and the total number of offspring per tube counted at this cut-off period. Since we could not dissect the tubes during the trial to visualize offspring development, this statistic enabled us to estimate the number of offspring that emerged from only the first generation, without including offspring from the second generation, so that we could compare brood sizes between temperature trials ("Offspring per colony"; Table 2.1). The average number of KSHB that emerged daily at each temperature was also calculated ("Daily emergence"; Table 2.1). Adult male emergence was recorded for all colonies to determine if their emergence was affected by ambient temperature.

Statistical Analysis & Modeling. Statistical analyses were performed using R v3.4.3 (R Core Team 2017). The number of days until first offspring emergence was

normally distributed at all temperatures except 24°C, which had one high-value outlier (Table S2.1). This outlier likely represented a foundress that had not mated prior to emergence, and thus had to produce haploid sons to mate with before producing daughters. After removing this outlier, all emergence data was normally distributed (Shapiro-Wilk test, $\alpha = 0.05$; $P > 0.05$). The effect of temperature on emergence time and number of offspring was determined using one-way ANOVA. Multiple comparisons were performed using post-hoc Tukey's HSD.

Mean emergence rate, which was calculated as the inverse of mean days until emergence, increased linearly from 18-30°C, and was modeled in this range using linear regression. Using this model, we calculated the lower temperature threshold (T_{min}) for KSHB using the equation $T_{min} = -a/b$ of the regression line (Campbell et al. 1974). To predict the optimum temperature (T_{opt}) and upper temperature threshold (T_{max}) for KSHB, we used formulae from Briere et al. (1999). The Briere model is a non-linear model that has been shown to be a reliable predictor of temperature-dependent rates in arthropods (Ganjisaffar and Perring 2015, Lee et al. 2018, Schoeller and Redak 2018, McCalla et al. 2019), and was used previously to model PSHB development (Umeda and Paine 2018). To find T_{max}, we used the formula:

$$\textbf{(1)}\ R(T) = aT(T - T_{min})(T_{max} - T)^{1/2},$$

where R is the developmental rate (here, the mean emergence rate) as a function of a constant temperature T, and a is an empirical constant. We also estimated T_{opt} using the formula:

(2) $$T_{opt} = \frac{(2mT_{max})+(m+1)T_{min}+[(4m^2{T_{max}}^2)+(m+1)^2{T_{min}}^2-4m^2T_{min}T_{max}]^2}{4m+2}$$

KSHB emergence at each temperature trial was also modeled using 3- and 4-parameter Gompertz, Weibull 1, and Weibull 2 functions, using the "drc" package in R (Ritz et al. 2015):

(3) Gompertz: $f(x) = c + (d - c)(\exp(-\exp(b(x - e))))$

(4) Weibull 1: $f(x) = c + (d - c)(\exp(-\exp(b(\log(x) - \log(e)))))$

(5) Weibull 2: $f(x) = c + (d - c)(1 - \exp(-\exp(b(\log(x) - \log(e)))))$,

where x = proportion KSHB emerged, b = rate of exponential increase (if larger, this parameter indicates more synchronous emergence), e = time in DD at 50% emergence, and c and d are model parameters. Model fit was assessed using the Akaike information criterion (AIC) and residual sum of squares (RSS); a model was said to be the best fit when these two statistics were minimized.

Finally, the time in cumulative degree-days (CDD) until first offspring emergence was calculated for all temperature trials using the formula $CDD = \Sigma\ T - T_{min}$. Because

KSHB females do not always emerge immediately after completing their development, we used the lowest CDD value across all trials to estimate the thermal constant (K).

Environmental Analysis. Publically-available weather data was collected from the California Irrigation Management Information System (CIMIS; State of California 2018) for the year 2018. Daily weather data were downloaded from the following CIMIS weather stations in California: Station #153 in Escondido (33.081050 N, -116.9757 W); Station #160 in San Luis Obispo (35.335261 N, -120.73588 W); Station #107 in Santa Barbara (34.437353 N, -119.73742 W); and Station #184 in San Diego (32.729578 N, -117.13934 W). These stations were chosen because KSHB has established or been collected in these locations. On days when data were not available from these stations, temperature data was taken instead from the following stations: for San Luis Obispo, Station #232 in Santa Maria (34.913472 N, -120.46478 W); for Santa Barbara, Station #231 in Lompoc (34.672222 N, -120.51306 W); and for Escondido and San Diego, Station #147 in Otay Lake (32.628208 N, -116.93928 W).

For each day, the maximum and minimum air temperatures were used to calculate the number of DD accumulated. CDD were calculated using the sine wave method in the program DEGDAY (Higley et al. 1986), using our model-derived estimates of T_{max} and T_{min}. The CDD accumulated in 2018 was then divided by K, which was found through our temperature cabinet experiments, to estimate the annual number of KSHB generations that would occur in each of these invaded locations in California.

RESULTS

Temperature Study. Temperature had a significant effect on the number of days until offspring emergence ($F = 64.48$, $df = 6$, $P < 0.001$; Figs. 2.2, S2.1), as well as the number of offspring that emerged per colony ($F = 4.992$, $df = 6$, $P < 0.001$; Fig 2.3, Table S2.2) and the number of offspring that emerged daily ($F = 109.2$, $df = 6$, $P < 0.001$; Table 2.1, Fig. S2.1). As a general trend, number of days until emergence decreased, and the number of successful colony tubes increased, as temperature increased up to 30°C (Table 2.1, S2.1; Fig. S2.1). The earliest emergence of offspring occurred at 30°C (28 days ± 2.65; Fig. 2.2, Table 2.1); however, there was no significant difference in emergence times from the 24, 26, 28, and 30°C trials ($P > 0.05$). The latest emergence occurred at 16°C (111 days ± 41) and was not significantly different from 18°C ($P = 0.972$), although days until emergence differed significantly between 18°C and 20°C ($P < 0.001$) and 20°C and 24°C ($P < 0.001$). The most synchronous emergence occurred at 28°C (Fig. S2.1, Table S2.5). Temperature did not have a significant effect on the number of males that emerged ($F = 1.211$, $df = 5$, $P = 0.313$; data not shown).

The highest percentage of colony tubes that produced emerging offspring occurred at 26°C and 28°C (58% and 61%, respectively; Table 2.1), and a significant decrease occurred at higher temperatures ($\chi^2 = 56.51$, $df = 7$, $P < 0.001$). Offspring emerged from only three tubes at 30°C, and no offspring emerged at 32°C. At 16°C very few offspring developed overall, with low colony success ($N = 2$). When the 16°C tubes were dissected, only one still contained offspring (2 adult females, 1 third instar female larva, and 1 egg). The majority of colony tubes were unsuccessful, showing signatures of

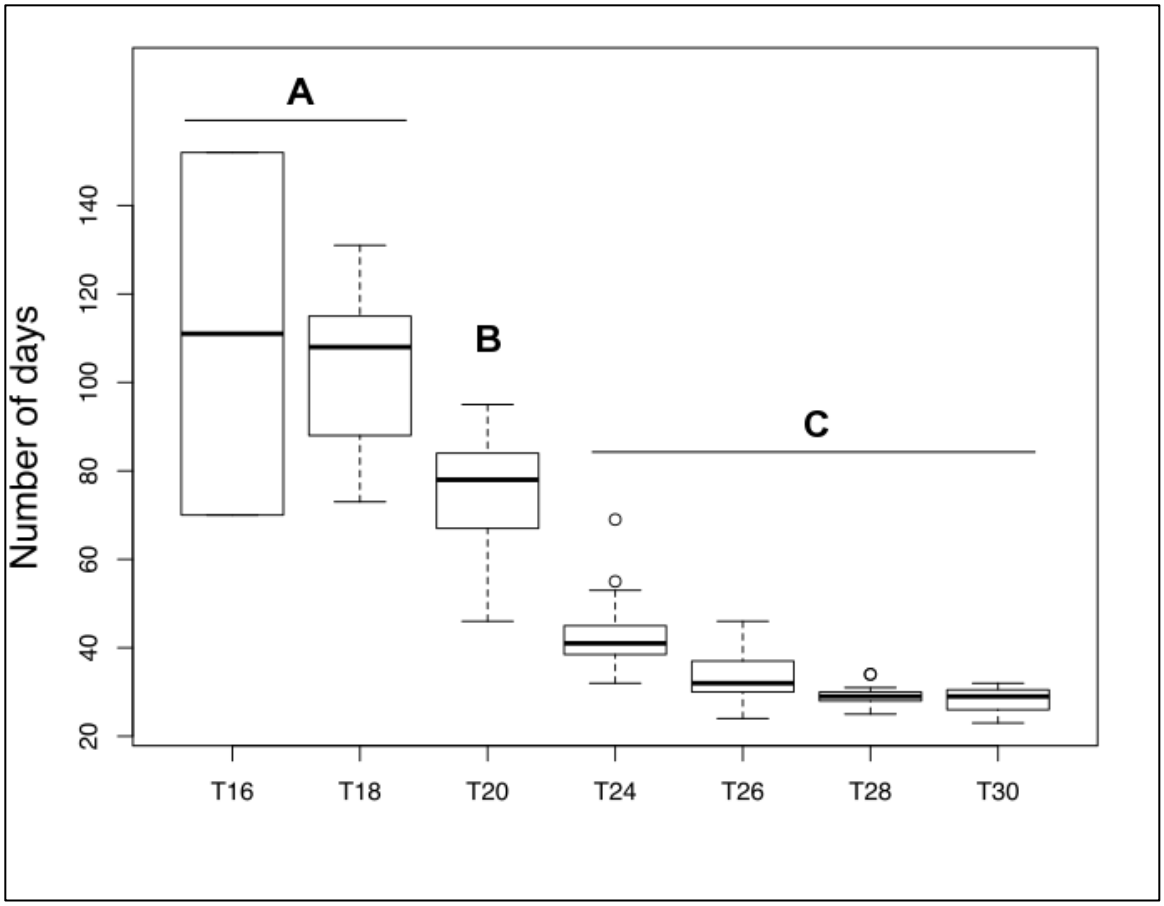


Fig. 2.2 Number of days until offspring emergence for each temperature trial. Box plots show sample minimum and maximum (horizontal lines at the bottom and top of each plot, respectively) and median (heavy line inside of box). Upper and lower quartiles are represented by the upper and lower limits of each box, respectively. Data points that fall outside of the quartile ranges are denoted as open circles. Letters indicate significance at $\alpha=0.05$.

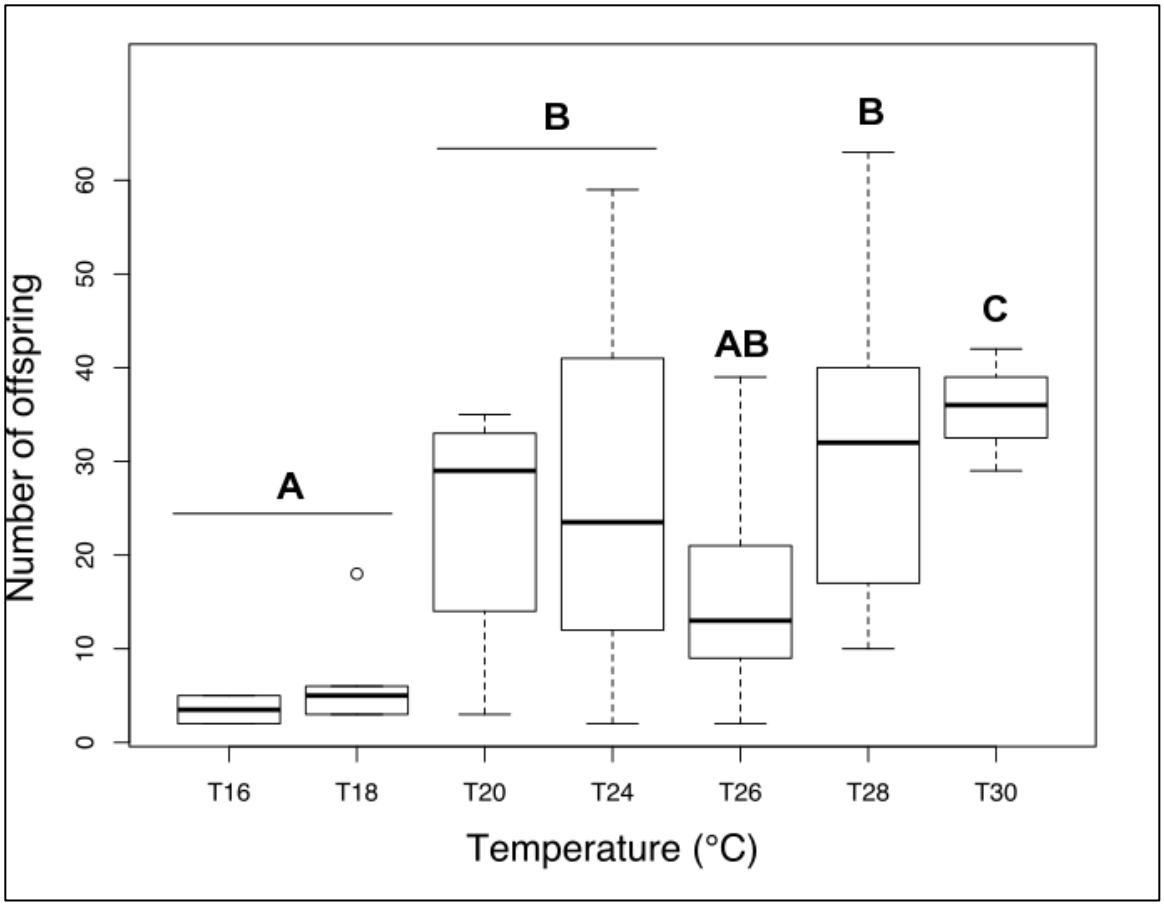


Fig. 2.3 Number of offspring per colony for each temperature trial. Box plots show sample minimum and maximum (horizontal lines at the bottom and top of each plot, respectively) and median (heavy line inside of box). Upper and lower quartiles are represented by the upper and lower limits of each box, respectively. Data points that fall outside of the quartile ranges are denoted as open circles. Letters indicate significance at α=0.05.

Table 2.1. Percentage of successful colony tubes (N) and mean ± SE values for days until emergence, offspring per colony tube, and offspring emerged per day at each temperature. Letters next to values indicate significant contrasts at $\alpha = 0.05$.

Temp. (°C)	N[a] (%)	Days until emergence	Offspring per colony	Daily emergence
16	2 (5.6%)a	111 ± 41a	3.5 ± 1.5a	0.061 ± 0.026a
18	5 (13.9%)a	103 ± 10.2a	7 ± 2.81a	0.322 ± 0.055a
20	13 (36.1%)b	75.2 ± 3.94b	28.54 ± 3.19b	2.7 ± 0.298b
24	20 (55.6%)c	43 ± 1.86c	30.95 ± 4.44b	10.17 ± 1.02c
26	21 (58.3%)c	33.1 ± 1.25c	16.76 ± 2.48ab	8.09 ± 0.882c
28	22 (61.1%)c	30.7 ± 1.61c	33.14 ± 3.91b	17.51 ± 1.81d
30	3 (8.3%)a	28 ± 2.65c	43 ± 2bc	3.15 ± 0.461b
32	0 (0.0%)a	N/A	N/A	N/A

[a] N = number of successful colony tubes from initial 36

failed gallery establishment by the foundress and no signs of offspring. Results were similar for the 18°C tubes, with only three tubes still containing live beetles (1 third instar female larva, 1 adult female, and 1 adult female, respectively). When the 32°C tubes were dissected, one tube contained two small larvae, and no other tubes contained offspring.

Environmental Analysis and Modeling. Our linear model was a good fit for the mean emergence rate from 18-30°C ($F = 227.9$, $df = 5$, adjusted $R^2 = 0.974$, $P < 0.001$). The equation of the regression line was found to be $y = -0.027x + 0.002$, and we found T_{min} to be 12.77°C. Using the Briere model formulae, our estimates of T_{max} and T_{opt} were 31.99°C and 28.04°C, respectively (Table 2.2).

For the sigmoid models, a 4-parameter model was the best fit, regardless of whether Weibull or Gompertz. 3-parameter models gave slightly better fit for some

Table 2.2 Critical temperatures and development time in degree-days (K) reported for members of the *Euwallacea fornicatus* species complex.

Species	T_{min}	T_{opt}	T_{max}	K	Reference
TSHB	15	30	32	373	Walgama & Zalucki 2007
PSHB	13.34	27.51	33.08	398	Umeda & Paine 2018
KSHB	12.77	28.04	31.99	318	Present study

temperature trials that had more overall data, but there was no significant difference between fit of these models and the 4-parameter Weibull 1 (ANOVA p-value > 0.05, Table S2.4), so we used the latter model to standardize results (Fig. 2.4, Table S2.5). The 16°C and 32°C trials were excluded due to insufficient emergence data. The sigmoid models predicted the time in CDD at which 50% of KSHB had emerged (e, Table S2.5), with substantial variation between trials. K varied greatly between temperature trials (Table S2.3), an unsurprising result given that KSHB do not always emerge immediately post-development. Using the lowest value across trials, we found K to be 318 CDD. The number of CDD in 2018 in Escondido, San Diego, Santa Barbara, and San Luis Obispo ranged from 1334-2388. Using K = 318 DD, we predict between 4-8 generations of KSHB annually across their invasive range in California, based on 2018 temperatures (Table 2.3, Fig. 2.5).

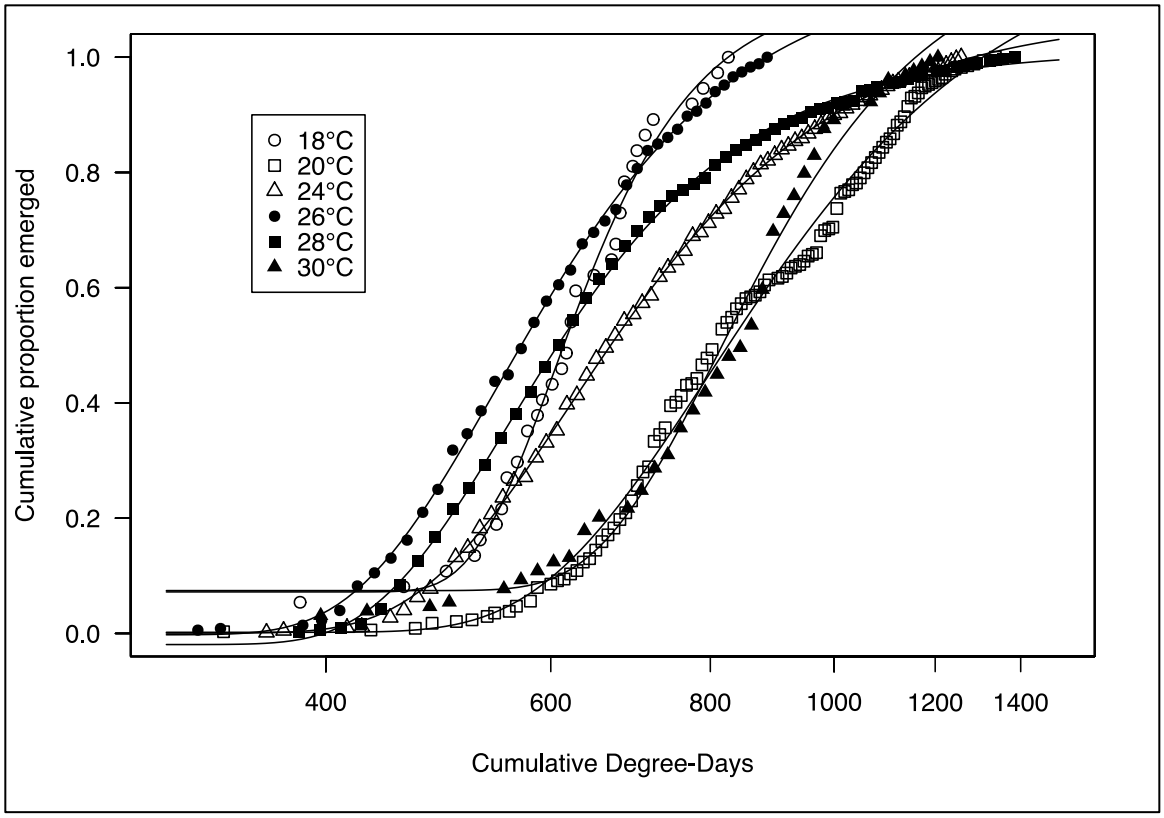


Fig. 2.4 Observed (shapes) and predicted (line) timing of offspring emergence, in degree-days, for KSHB reared under a series of constant temperature regimes.

Table 2.3 CDD and predicted number of KSHB generations in 2018 for four locations in California with invasive populations.

Location	CDD in 2018	Number of KSHB generations
San Luis Obispo	1333.9	4.19
Santa Barbara	1816.6	5.71
Escondido	2388.4	7.51
San Diego	2095.0	6.59

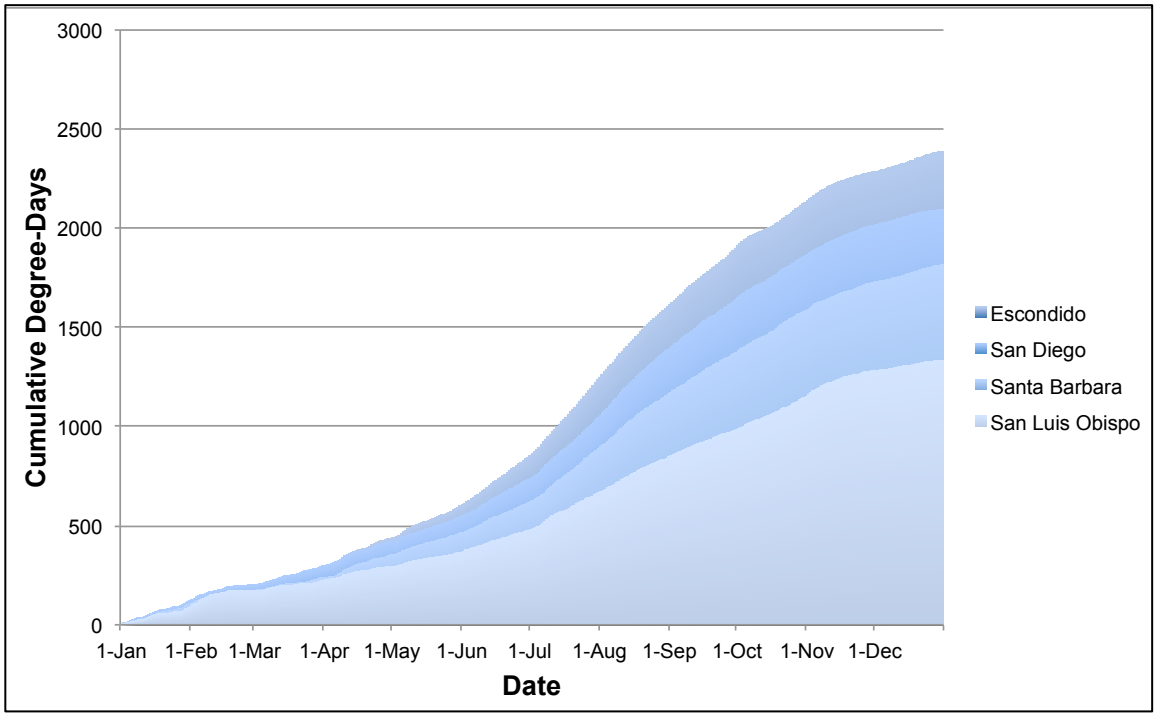


Fig. 2.5 Comparison of degree-day accumulation for the year 2018 in four locations with invasive KSHB populations in California.

DISCUSSION

Based on our experimental trials, the optimal temperature for KSHB development and emergence is between 26-30°C (Table 2.1, Fig. 2.2). The highest reproductive success (number of successful tubes and mean offspring per foundress) and emergence was observed at 28°C. This result is compatible with our model-derived T_{opt} of 28.04°C. Our rearing trials produced no successful tubes at 32°C, supporting our T_{max} estimate of 31.99°C. Overall, we obtained similar values for critical temperature thresholds to what has been reported for other members of the *Euwallacea fornicatus* species complex (Table 2.2).

From our model estimate of T_{min}, we predict that KSHB may develop at temperatures as low as about 13°C. However, our experimental trials revealed decreased emergence at temperatures below 20°C (Table 2.1, Fig. 2.2). The thermal constant K for KSHB was found to be 318 DD, which is much lower than what has been found for PSHB and TSHB (Table 2.2). This is surprising because K was derived from developmental data in previous studies but emergence data in the present study, so we expected our K to be an overestimate. However, interspecific differences in thermal requirements are also possible. Using K, we predict between 4-8 generations of KSHB annually in its invasive range in California, based on temperature data from four locations in 2018 (Table 2.3).

The 30°C trial had the highest mean offspring per foundress and the earliest emergence, but had significantly fewer tubes that produced offspring (Table 2.1). A possible explanation for this pattern is that KSHB is approaching the upper threshold of

emergence and development, and has a lower chance of reproducing successfully at this temperature than at 28°C. An alternative, but not exclusive, explanation is that at 30°C, the symbiotic fungi of KSHB do not grow as efficiently, are more likely to be outcompeted by free-living opportunistic fungi, or both. The production and development of ambrosia beetle offspring are dependent on the availability of the beetles' symbiotic fungi; if these fungi are not able to grow, or are inhibited by competing fungi, then offspring production will be low (Beaver 1989). At around 28°C, the growth rate of *F. kuroshium* and *G. kuroshium* may be maximized, but above this temperature other antagonistic fungi may grow faster. Similarly, slow fungal growth could explain the reduced success of KSHB at lower temperatures. Umeda and Paine (2018) found that PSHB could survive for at least 200 days at 15°C, but produced no viable offspring. At 16°C, we found low offspring production, but extensive gallery excavation by KSHB foundresses. While the foundresses may have been vigorous enough to excavate galleries, the cold temperatures may have impeded fungal growth and prevented reproduction.

We did not address the effects of precipitation, humidity, solar radiation, and other environmental factors in the present study. These factors likely all contribute to the emergence and flight of KSHB, and should be incorporated into future models. Additionally, analyses of gallery microclimate and within-tree conditions may help to couple development time with emergence and flight. It is known that temperature conditions within a host tree are typically more stable year-round compared to outside air temperatures (Vermunt et al. 2012). During summer, the temperature within the tree is likely cooler than the outside temperature, whereas during winter it may be warmer inside

the tree than outside. This would affect the number of generations per year accordingly. However, many biotic and abiotic factors dictate the within-tree microclimate that wood-boring beetles experience (Bartos and Amman 1989). In addition, host tree preferences vary depending on ambrosia beetle species and niche, for example whether the ambrosia beetle is a primary or secondary invader. Primary ambrosia beetles, such as members of the *Euwallacea fornicatus* species complex, represent the minority of ambrosia beetle species and attack live, healthy trees, whereas secondary ambrosia beetles colonize trees that are already dead or dying (Paine et al. 1997). Primary and secondary ambrosia beetles are therefore likely to experience radically different within-tree conditions that dictate their development and emergence. Because of this, analysis of within-tree conditions should be performed on a species-specific basis.

APPENDIX

Table S2.1. Onset of offspring emergence for all colonies for each temperature trial. Values indicate number of days post-introduction of foundress.

	Temperature trial (°C)						
Tube No.	**16**	**18**	**20**	**24**	**26**	**28**	**30**
1	70	73	46	32	24	25	23
2	152	88	60	33	24	26	29
3		108	65	37	25	26	32
4		115	67	37	29	27	
5		131	67	37	29	28	
6			72	40	30	28	
7			78	40	31	28	
8			78	40	31	29	
9			81	40	32	29	
10			84	40	32	29	
11			91	42	32	29	
12			93	42	32	29	
13			95	43	34	29	
14				45	34	29	
15				45	35	30	
16				45	37	30	
17				45	37	31	
18				53	38	31	
19				55	41	31	
20				69	42	34	
21					46	34	
22						63	

Table S2.2. Summary statistics of offspring counts across all colony tubes for each temperature trial.

Temperature (°C)	Min	1st Quartile	Median	Mean	3rd Quartile	Max
16	2	2.25	2.5	2.5	2.75	3
18	3	4.0	5.0	7.2	6.0	18
20	2	14.0	29.0	22.69	33.0	35
24	3	12.5	23.5	26.6	40.5	59
26	2	9.0	13.0	17.1	21.0	39
28	10	18.0	33.0	31.81	40.0	63
30	29	32.25	35.5	35.5	38.75	42
32	0	0	0	0	0	0

Table S2.3. Cumulative degree-days (CDD) until first offspring emergence for each temperature trial (16°C and 32°C trials excluded due to insufficient data).

Temperature (°C)	CDD
18	381.79
20	332.58
24	359.36
26	317.52
28	380.75
30	396.29

Table S2.4. Best-fit model statistics for each temperature trial.

Temperature (°C)	Best fit model	AIC	RSS	p-value[a]
18	4-parameter Gompertz	-126.97	0.027	1.00
20	3-parameter Weibull 1	-461.65	0.026	1.00
24	3-parameter Weibull 1	-546.67	0.006	1.00
26	3-parameter Weibull 1	-265.54	0.009	0.609
28	4-parameter Weibull 1	-416.13	0.008	N/A
30	4-parameter Weibull 2	-193.31	0.019	1.00

[a] P-value from ANOVA between best-fit model and 4-parameter Weibull 1 model.

Table S2.5. Model estimates of parameters (using 4-parameter Weibull 1) for each temperature trial.

T (°C)	AIC	RSS	df	b	c	d	e
18	-125.48	0.027	26	-6.85 ± 0.507	0.072 ± .018	1.12 ± 0.37	538.09 ± 3.76
20	-459.22	0.026	100	-3.29 ± 0.148	0.002 ± 0.010	1.22 ± 0.031	735.39 ± 6.53
24	-543.53	0.006	70	-3.66 ± 0.03	0.001 ± 0.003	1.07 ± 0.004	589.28 ± 1.05
26	-263.84	0.009	37	-3.96 ± 0.09	-0.002 ± 0.005	1.16 ± 0.012	520.25 ± 1.74
28	-416.13	0.008	58	-4.16 ± 0.06	-0.019 ± 0.006	1.01 ± 0.003	534.25 ± 1.57
30	-145.62	0.035	35	-4.60 ± 0.47	0.074 ± 0.015	1.20 ± 0.055	785.41 ± 9.16

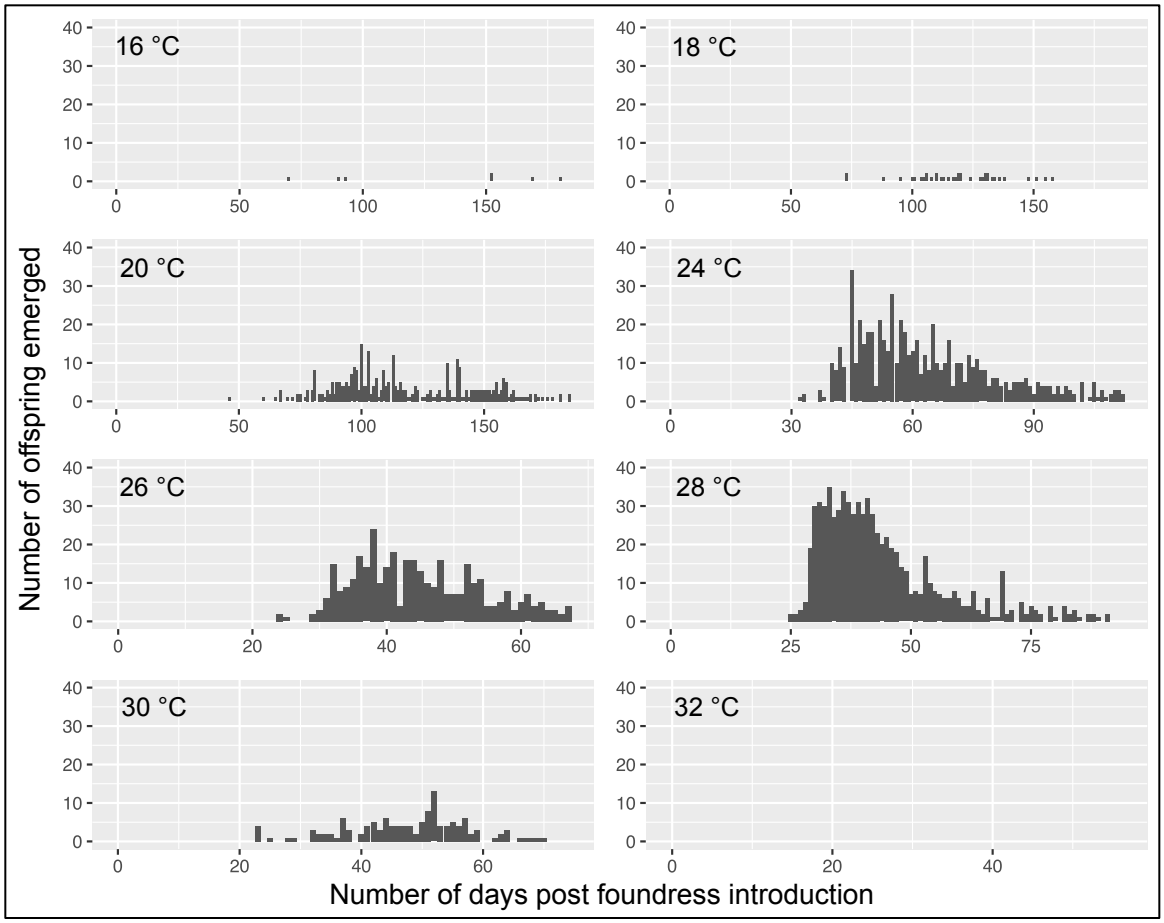


Fig. S2.1 Total offspring emergence per day for each temperature trial, with the number of days since the foundress was introduced to the colony tube on the x-axis.

Chapter III. Fungal symbionts of the polyphagous and Kuroshio shot hole borers (Coleoptera: Scolytinae, *Euwallacea* spp.), invasive ambrosia beetles in California, can support both beetle systems on artificial media

ABSTRACT

The specificity of ambrosia beetle-fungus relationships is unknown for the vast majority of ambrosia symbioses, yet has important implications for the success of these beetles and fungi in their surrounding environments. The polyphagous shot hole borer and the Kuroshio shot hole borer (*Euwallacea* spp.) are invasive ambrosia beetles that exist sympatrically in California, and together with their fungi cause the plant disease Fusarium Dieback in a variety of hosts. The potential for the two beetle species to exchange symbionts is unknown, but has important ecological consequences. Here we perform symbiont switching experiments, to determine if the polyphagous and Kuroshio shot hole borers can survive and reproduce on each other's symbiotic fungi. Aposymbiotic foundresses were assessed on their ability to reproduce on each of the beetles' primary (*Fusarium* spp.) and auxiliary (*Graphium* spp., *Paracremonium* sp.) fungal symbionts, grown on sawdust-based artificial media. Offspring were collected to confirm which fungi were present in the different sexes and life stages. Additionally, the ability of aposymbiotic and non-aposymbiotic foundresses to survive and reproduce on non-ambrosial *Fusarium* spp. was tested. We found that reproduction is maximized on the beetles' primary *Fusarium* symbionts, regardless of whether a beetle was paired with its own *Fusarium* symbiont or its congener's, indicating that there is potential for

symbiont switching in natural populations of these beetles. The addition of auxiliary fungi to treatments containing *Fusarium* spp. had no effect on overall offspring production, and females that were fed solely on auxiliary fungi produced significantly fewer offspring. Females that were fed non-ambrosial *Fusarium* spp. produced significantly fewer offspring than when reared on symbiotic *Fusarium*, and this was especially pronounced in aposymbiotic females. From our experiments, the putative roles of the primary versus auxiliary fungal symbionts, as well as the implications of these roles for beetle reproduction and development in their surrounding environment, are discussed.

INTRODUCTION

Ambrosia beetles in the subfamily Scolytinae (Coleoptera: Curculionidae) represent one of the most diverse and evolutionarily successful clades of insects, due in part to their mutualistic symbioses with fungi (Kirkendall et al. 2015). They are predominantly xylomycetophagous and rely on fungal symbionts to complete their life cycle in the trunks and branches of trees (Beaver 1989). Their ability to farm these "ambrosia" fungi within trees has made them a concern when introduced into non-native habitats, where some species have been reported to cause considerable economic damage to local flora (Hanula et al. 2008, Cognato et al. 2015, O'Donnell et al. 2016, Hulcr and Stelinski 2017), as well as agriculturally important commodities (Danthanarayana 1968, Mendel et al. 2012, Eskalen et al. 2013). The evolution of specialized glandular organs called mycangia (Francke-Grosmann 1956, Batra 1963) enables the beetles to vector

nutritious fungal propagules from host to host, facilitating a mutualistic symbiosis between both partners. Mechanical injury to wood through boring activity enables the rapid penetration of fungal propagules and the initiation of fungal gardens, which the beetles actively cultivate and feed upon (Batra 1985). Ambrosia beetles have historically been thought to have species-specific associations with ambrosia fungi (Batra 1963), but recent evidence suggests that these insect-fungus interactions can be promiscuous among some mutualisms (Carrillo et al. 2014, Kostovcik et al. 2015).

Ambrosia beetles in the *Euwallacea fornicatus* species complex (Cooperband et al. 2016, Stouthamer et al. 2017) are native to Southeast Asia (Rabaglia et al. 2006) and are considered pests of economic importance in many areas they have invaded. The tea shot hole borer (TSHB) *E. perbrevis* Schedl (formerly *E. fornicatus* Eichoff; Smith et al. 2019), with its fungal mutualist *Fusarium ambrosium* (C. H. Gadd & C. A. Loos)(Gadd and Loos 1946), has been a significant pest of tea in India and Sri Lanka for over a century (Gadd 1941, Danthanarayana 1968). The polyphagous shot hole borer (PSHB) *E. fornicatus* Schedl vectors the plant disease Fusarium Dieback (FD) within susceptible host trees in California (Eskalen et al. 2013), Israel (Mendel et al. 2012), and South Africa (Paap et al. 2018), through the spread of its primary symbiont *Fusarium euwallaceae* (S. Freeman, Z. Mendel, T. Aoki & O'Donnell), as well as the auxiliary fungi *Graphium euwallaceae* (M. Twizeyimana, S. C. Lynch & A. Eskalen) and *Paracremonium pembeum* (S. C. Lynch & A. Eskalen) (Eskalen et al. 2013, Lynch et al. 2016). The closely-related Kuroshio shot hole borer (KSHB) *E. kuroshio* Gomez & Hulcr (Gomez et al. 2018) has also established in California and been found to cause FD

through association with *Fusarium kuroshium* (F. Na, J. D. Carrillo & A. Eskalen) and *Graphium kuroshium* (F. Na, J. D. Carrillo & A. Eskalen) (Na et al. 2018). These three *Fusarium* species belong to the monophyletic Ambrosia *Fusarium* Clade (AFC) within the *F. solani* species complex (FSSC) clade 3 (Kasson et al. 2013, O'Donnell et al. 2015). The dominance of AFC members within mature female mycangia suggests that they are the primary symbionts of these beetles.

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

Obligate specificity of PSHB to *F. euwallaceae* was previously investigated; it was found that larvae placed on petri dishes colonized with *F. ambrosium* (the symbiont of TSHB)*, F. mangiferae,* and *F. oxysporum* (both non-ambrosial fungi) all had significantly higher mortality than when fed *F. euwallaceae* (Freeman et al. 2013a). However, phylogenetic analyses from a later study showed that a *Euwallacea* sp. recovered from Florida was associated with two different AFC members: *Fusarium* sp. [AF-6] from AFC clade B and *Fusarium* sp. [AF-8] from AFC clade A (O'Donnell et al. 2015). Early work done in this field revealed that ambrosia beetles may not feed solely on a primary symbiont throughout its life cycle, but may also feed on auxiliary fungi (Batra 1967). Thus, their diet likely consists of the entire microbial complex present in the fungal garden, including bacteria and other fungi, although the proportions of various fungi and bacteria consumed can vary with the life stage of the beetle, age of the gallery, and host tree species (Batra 1966, Haanstad and Norris 1985, Biedermann et al. 2013). *In vitro* diet studies help to reveal the function and importance of such auxiliary fungi on ambrosia beetle survival and reproduction. In PSHB, for example, Freeman et al. (2015) showed that *F. euwallaceae* and *G. euwallaceae* are suitable food sources for larvae, while *P. pembeum* alone was not sufficient for larval development.

The objectives of this study were to (i) investigate fungal symbiont fidelity of aposymbiotic PSHB and KSHB on both “natural” (their own) and “alternate” (their congener’s) diets of *F. euwallaceae*, *G. euwallaceae*, *P. pembeum, F. kuroshium*, and *G. kuroshium*, given in individual and combination treatments; (ii) evaluate the occurrence and proportion of each symbiont in different sexes and life stages of the beetles; and (iii)

compare offspring counts of PSHB and KSHB across all treatments as a measure of fitness. We also (iv) investigate the ability of both untreated and aposymbiotic PSHB to survive on non-ambrosial *F. solani* and *F. oxysporum* recovered from avocado wood in California, by counting offspring and recovering fungal colony forming units (CFU) from female mycangia.

MATERIALS AND METHODS

Beetle collection and rearing. PSHB and KSHB were collected in California, USA in 2014 from infested avocado groves (*Persea americana* Mill cv. "Hass") in Los Angeles and San Diego Counties, respectively. The beetles were collected live from funnel traps baited with quercivorol (Dodge et al. 2017) or from brood galleries by dissecting wood from infested host trees, and confirmed as PSHB and KSHB through quantitative polymerase chain reaction (qPCR) analysis as described by Rugman-Jones and Stouthamer (2016). Beetles were surface disinfested with 70% ethanol for 5 s, dried on filter paper, and introduced into artificial sawdust-based media (75 g avocado sawdust, 20 g agar, 10 g sucrose, 5 g casein, 5 g starch, 5 g yeast, 1 g Wesson's salt mixture, 2.5 ml wheat germ oil, and 5 ml 95% ethanol in 500 ml of distilled water) modified from Peer and Taborsky (2004). Sawdust was generated from avocado logs >10 cm in diameter that were collected from uninfested Hass cultivars at the University of California, Riverside (UCR) Agricultural Operations; wood was collected using a chop saw and stored at -20ºC until ready for use. Once combined, the artificial media was thoroughly mixed, autoclaved for 30 min at 121°C, and poured hot, to reduce viscosity, into 50 ml

Falcon™ conical centrifuge tubes (BD Biosciences, Bedford, MA, USA) up to the 25 ml mark. Tubes were allowed to dry in a sterile laminar flow hood to evaporate condensed moisture, and then capped until use. Before introducing beetles to the tubes, holes were poked ~2 mm deep into the media using a sterilized 2 mm diameter metal rod, to facilitate boring activity. After beetles were introduced, the caps were left loose to allow gas exchange and kept at a constant temperature and relative humidity of 24°C and ~40%, respectively. Beetle colonies were propagated in the UCR Insectary and Quarantine Facility, and subsequent generations were used in fungal diet studies.

Preparation of aposymbiotic PSHB and KSHB. It has been shown that adult ambrosia beetles are generally free of their fungal symbionts (i.e., aposymbiotic) when eclosion occurs outside of their natal gallery (Batra 1966). To obtain aposymbiotic PSHB and KSHB, 20-30 fertilized female beetles from individual colonies were introduced into individual sawdust media tubes and allowed to establish galleries for 21-25 days, or until numerous pupae were visible in some of the tubes. Tubes were dissected, and viable F1 female pupae were removed using a fine miniature paint brush that was cleaned with 95% ethanol and allowed to dry prior to handling. The pupae were placed in clean glass petri dishes lined with sterile Kimwipes® (Kimberly-Clark, Irving, TX, USA) that were moistened with sterile distilled water. The dishes were wrapped with Parafilm® (Neenah, WI, USA) to prevent desiccation of pupae, and replenished with sterile distilled water as needed. No more than 15 pupae were added to each dish, to prevent overcrowding and potential loss from mycosis. Eclosion occurred after 1-7 days, and teneral virgin adult females were surface sterilized with modified White's solution (1 g $HgCl_2$, 6.5 g NaCl,

1.25 ml HCl, and 250 ml 95% ethanol per 1 L distilled water; Kopper et al. 2003). Prior to *in vitro* diet experiments, symbiotic fungi were quantified from aposymbiotic virgin females versus non-virgin females by plating contents from the head and body onto fresh potato dextrose agar (PDA; BD Difco, Sparks, MD, USA) media plates amended with 0.01% tetracycline hydrochloride (PDA-t). This verified that the mycangia were free of fungal symbionts, and the bodies nearly so (Table 3.1); however, the complete removal of fungi from female bodies is virtually impossible, and was not achieved during our experiments.

Preparation and dissection of experimental media tubes. Fungal isolates used in this study were *F. euwallaceae* (NRRL 62626), *G. euwallaceae* (CBS 140035), and *P. pembeum* (CBS 140042) previously acquired from PSHB (Freeman et al. 2013b, Lynch et al. 2016), and *F. kuroshium* (CBS142642) and *G. kuroshium* (CBS142643) previously acquired from KSHB (Na et al. 2018). The plant pathogen *Lasiodiplodia citricola* (Botryosphaeriaceae; UCR6961), recovered from an avocado branch canker near shot hole borer galleries in San Diego County, was used as a negative control. To prepare the fungal treatments, spore suspensions from isolates of *F. euwallaceae, F. kuroshium, G. euwallaceae, G. kuroshium,* and *P. pembeum* were made by scraping one-week old

Table 3.1. Fungal symbionts recovered from untreated vs. aposymbiotic females

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

a All individuals adult PSHB females from lab colonies reared on artificial media

sporulating cultures with a sterile inoculating loop and streaking it onto fresh plates of PDA-t. Plates were grown at 25°C for three days, after which 5 ml of sterile distilled water was added to each plate and agitated with sterile bent glass rods to release the spores into suspension. The suspension was passed through a layer of sterile Kimwipe® to remove large mycelial fragments. All spore suspensions were quantified using a Bright-Line hemocytometer (Hausser Scientific, Horsham, PA, USA) and diluted to $1x10^6$ spores/ml. Fresh sawdust media tubes were inoculated by depositing a total of 10 µl of spore suspension onto the surface of the media and spreading with a sterile glass pestle to distribute spores evenly across the surface. Fungal spore suspensions of beetle symbionts were confirmed as the desired species through qPCR using species-specific primers and probes described in Carrillo et al. (2019). Fungal plugs (3 mm) of 1-week old *L. citricola* were taken with a cork borer and placed mycelium side down on the media surface.

The fungal diet treatments were as follows: *F. euwallaceae, G. euwallaceae, F. euwallaceae* + *G. euwallaceae* (5 µl of each suspension), *P. pembeum, F. kuroshium, G. kuroshium, F. kuroshium* + *G. kuroshium* (5 µl of each suspension), *L. citricola* (negative control), and a non-treated control (NTC) consisting of an uninoculated artificial media tube. After fungal inoculation, the tubes were incubated at 25°C for three days before introducing the aposymbiotic virgin female beetles (one per tube). The experiment was designed using randomized complete blocks, where individual beetles of each species were randomly assigned to the nine treatments within each block; this was replicated five times for a total of 45 observations per beetle species per trial. Beetles in the genus

Euwallacea undergo arrhenotokous parthenogenesis (Kirkendall et al. 2015); thus a virgin female will produce only male F1 offspring, which she can then mate with to produce F2 daughters. After ~80 days, when F2 generation females were present, the diet tubes were dissected and all life stages (eggs, larvae, pupae, and adults) were counted. Pupae and adults were sexed, and larvae, pupae, and adult males and females were sampled (2 of each per tube, when possible) to investigate the fungi present in different sexes and life stages. Offspring were quickly euthanized in 70% ethanol and immediately processed to recover fungi.

Isolation and quantification of fungal symbionts from offspring. Beetle offspring from experimental media tubes were surface sterilized by submersion in 70% ethanol, vortexed for 5 s, rinsed with sterile deionized water, and allowed to dry on sterile filter paper. All samples were processed individually except larvae, which were pooled into one sample (two larvae per sample). Additionally, adult female beetle heads were separated from the rest of the body using a sterile blade under a dissection microscope and processed separately to investigate differences between the female head (which contains the mycangia) and the female body (guts). All samples were macerated in 1.5 ml microcentrifuge tubes with sterile plastic pestles. The macerated samples were suspended in 1 ml of sterile 0.01% triton (except for adult female bodies, which were suspended in 200 µl) and further diluted to different concentrations depending on the sample type: 1:1 dilution for female heads, 3:1 dilution of entire males, larvae, and pupae, and no dilution for female bodies. 25 µl of suspension from each sample was pipetted onto three replicate petri dishes containing PDA-t and spread using sterile glass L-shaped rods. Plates were

incubated for two days at 25°C. One of the three plates was used to acquire 5 single-spore fungal colonies, sampled at random, which were transferred to individual plates containing ½ strength PDA (9.75 g PDA and 3.75 g agar in 500 ml distilled water), mycelium side down. After one week of incubation at 25°C, the resulting colonies were grouped by morphology and identified to genus (*Fusarium*, *Graphium*, or *Paracremonium*) based on previous descriptions of morphology on PDA (Freeman et al. 2013a, Lynch et al. 2016, Na et al. 2018). Representatives of each group of interest were identified to species using the qPCR assay described by Carrillo et al. (2019). The remaining two plates were allowed to grow for one week at 25°C, after which period the total CFUs were counted and distinguished, as above, based on previous descriptions of morphology on PDA. Information was recorded using custom-built forms on the iForm application (Zurion Software Inc., Herndon, VA, USA). Common contaminant fungi, such as *Penicillium* spp., *Aspergillus* spp., and *Rhizopus* spp., were not recorded.

Aposymbiotic vs. non-aposymbiotic female survival and reproduction on non-ambrosial *Fusarium* spp. Fungal isolates used in this experiment were *F. euwallaceae* (NRRL 62626), *F. solani* (UCR7265 and UCR7266, recovered from California sycamore (*Platanus racemosa*, Orange County) and avocado (San Diego County), respectively), and *F. oxysporum* (UCR3424 and UCR6846, recovered from avocado from Orange and Riverside Counties, respectively). To prepare the fungal treatments, spore suspensions were prepared from isolates following the methods described above and diluted to a final concentration of $1x10^6$ spores/ml. Fresh artificial media tubes were inoculated with 10 µl of spore suspension. The treatments were as

follows: *F. euwallaceae, F. solani, F. oxysporum*, and a non-treated control (NTC). Experimental design was a randomized complete block design where, within each block, the four treatments were applied to two groups of PSHB taken from colony: aposymbiotic virgin females, prepared as described above; and non-aposymbiotic (i.e., untreated) females that carried their full complement of natural symbionts. This was replicated five times for a total of 20 observations per beetle status per trial. Aposymbiotic virgin females were incubated for ~80 days to observe females in the F2 generation, while untreated females, which were already mated, were incubated for ~40 days to observe females in the F1 generation. All offspring of all life stages (eggs, larvae, pupae, and adults) were counted, sampled, surface sterilized, and processed as described above. In this experiment, the gallery wall was also sampled by opening the media to expose the inner gallery wall, rotating the tip of a sterile wooden toothpick 360° in the gallery, and suspending the toothpick in 1 ml sterile 0.01% triton. The beetle samples were diluted to the ratios reported in previous sections, and gallery samples were not diluted. 25 µl of suspension from each sample was pipetted onto three replicate PDA-t plates and spread using sterile glass L-shaped rods. Plates were incubated for one week at 25°C and total CFUs were counted. *F. euwallaceae, F. solani, and F. oxysporum* were distinguished using previously published descriptions of morphology (Leslie and Summerell 2006, Freeman et al. 2013b). In this experiment, fungal recovery from the female head (mycangia) was compared to that from the female body and gallery.

Statistical analysis. Statistical analyses were performed using R v3.4.3 (R Core Team 2017) or Stata (StataCorp 2017). Offspring count data was found in all cases to be

overdispersed (the variance exceeds the mean) using the package "COUNT" (Hilbe 2016) in R. Thus, a negative binomial regression was used to analyze offspring count data using the "MASS" package (Venables and Ripley 2002) in R. Analysis of Variance (ANOVA) was used to compare independent variables (beetle treatment, fungal treatment, block, trial) in the regression model using the *anova* function in R. Pairwise comparisons of offspring counts between PSHB and KSHB were performed by sample type (egg, larva, pupa, adult male, adult female) within each fungal treatment, as well as across all sample types and treatments using estimated marginal means (EMM) with the "emmeans" package (Lenth et al. 2018) in R. CFU spore count data was square-root transformed and analyzed using Poisson regression analysis, which was performed using a generalized linear model using the "MASS" package in R. Post-hoc analysis for CFU data was done by constructing a contrast matrix to compare all sample type responses to fungal treatments between PSHB and KSHB, then by using the "multcomp" package (Hothorn et al. 2008) in R with link function *glht* for comparison of recovered CFU counts between PSHB and KSHB based on the matrix. A multinomial logistic regression analysis was performed in Stata using the "mlogit" syntax to compare recovery of fungi from different offspring sample types within each fungal treatment. P-values for multinomial logistic regression were generated using Wald tests for each independent variable. All bar plots were generated using the "ggplot2" package (Wickham et al. 2016) in R. Negative control (*L. citricola*) and NTC data were only considered in total offspring analysis.

RESULTS

PSHB and KSHB reproduction on natural and alternate fungal diets.

ANOVA revealed a significant effect of fungal treatment ($\chi^2 = 294.59$, $df = 8$, $P < 0.001$), but not beetle treatment ($P = 0.189$), on total offspring counts (all life stages pooled; Fig. 3.1). The same result was found for eggs ($\chi^2 = 121.21$, $P < 0.001$), larvae ($\chi^2 = 236.17$, $P < 0.001$), pupae ($\chi^2 = 228.50$, $P < 0.001$), and adult females ($\chi^2 = 261.11$, $P < 0.001$) when separate ANOVAs were performed by sample type. There was a significant effect of both fungal treatment ($\chi^2 = 240.47$, $P < 0.001$) and beetle treatment ($\chi^2 = 15.83$, $P < 0.001$) on adult male counts, which were greater for PSHB than KSHB.

EMM analysis of the negative binomial regression model revealed that all fungal treatments that included either *F. euwallaceae* or *F. kuroshium*, considered the primary symbionts of PSHB and KSHB, respectively, had higher total offspring counts compared to treatments without the primary symbionts ($P < 0.05$; Fig. 3.1). Individual treatments of auxiliary fungi *G. euwallaceae, G. kuroshium,* and *P. pembeum* had offspring counts significantly greater ($P < 0.05$) than the negative control (*L. citricola*) and the NTC (Fig. 3.1). Offspring counts from combination treatments of *Fusarium* spp. + *Graphium* spp. were not significantly different from treatments that contained only *Fusarium* spp. ($P > 0.05$; Fig. 3.1). Offspring counts were broken down by sample type with five levels (adult females, adult males, pupae, larvae, and eggs). Direct comparisons between the two

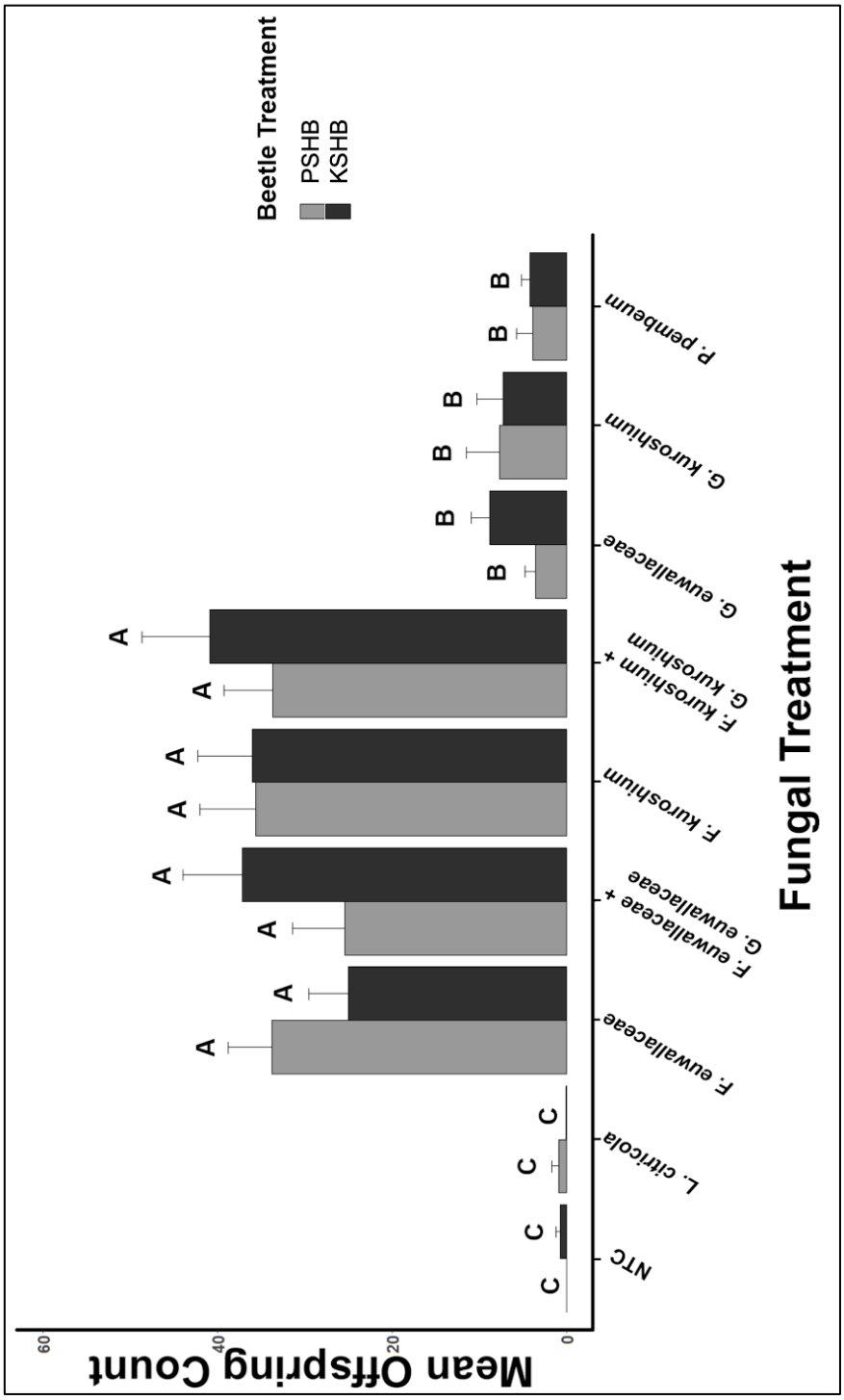


Fig. 3.1 Total offspring counts across all individuals within each fungal treatment group. Letters represent significant contrasts at $\alpha = 0.05$ using post-hoc EMM from negative binomial regression model.

beetle species across all sample type/fungus combinations showed significant differences ($P < 0.05$) only between eggs on the *F. euwallaceae* + *G. euwallaceae* treatment; adult males on the *F. kuroshium* and *G. euwallaceae* treatments; and adult females on the *G. euwallaceae* treatment (Fig. 3.2). Otherwise, there were no significant differences in offspring counts between PSHB and KSHB on any fungal treatment.

Fungal species recovery from different fungal treatments and offspring sample types. Overall proportions of the different fungal species recovered from beetles from each treatment confirm that they primarily consumed the fungi that were provided as diet, rather than carryover fungi from their natal gallery (Fig 3.3; Table S3.3). Analysis of regression coefficients showed that overall fungal recovery was similar between PSHB and KSHB within fungal treatments, with some exceptions (Table S3.1). Offspring sample types were also compared within each treatment (Table S3.2), to determine which fungi were most likely to be recovered from the different sexes and life stages. In the *F. euwallaceae* and *F. kuroshium* treatments, larvae were more likely than any other sample type to harbor auxiliary fungi based on recovered fungi (Table S3.2). When given combination *Fusarium* + *Graphium* treatments, female heads were more likely to harbor the *Fusarium* species provided than the *Graphium* species provided (Table 3.2); this was true for both PSHB and KSHB.

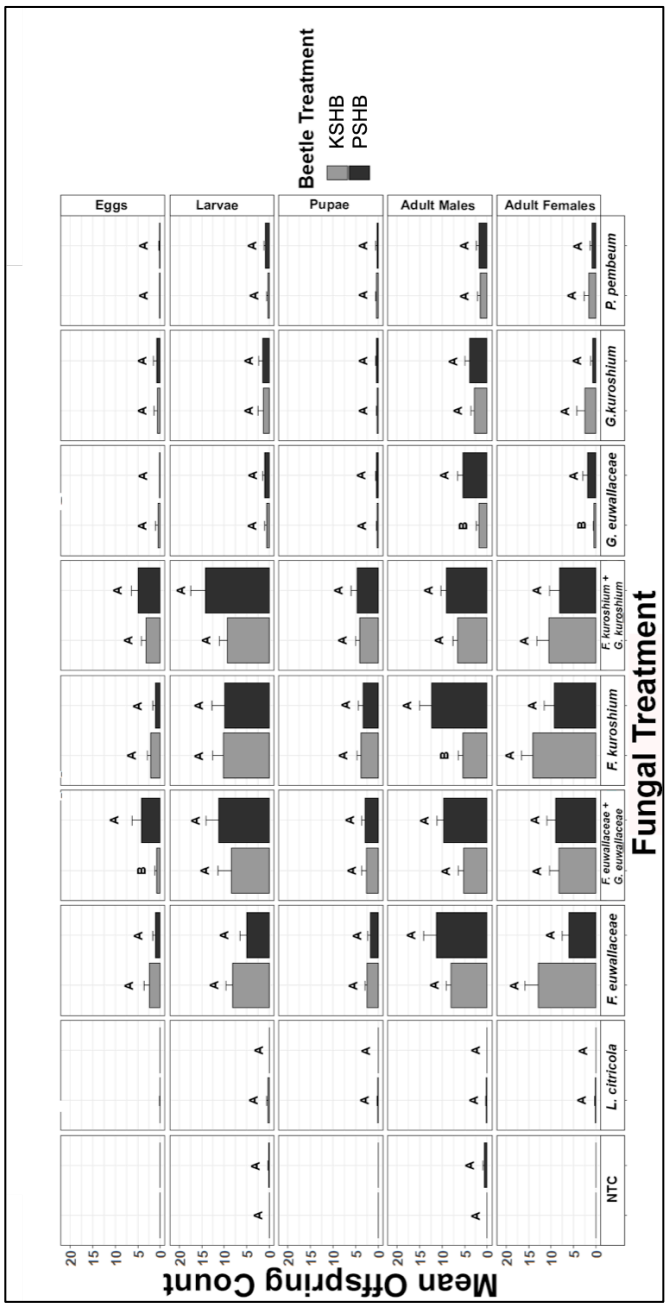


Fig. 3.2 Offspring counts split by sample type within each fungal treatment group. Letters represent significant contrasts within each facet at $\alpha = 0.05$ using post-hoc EMM from negative binomial regression model.

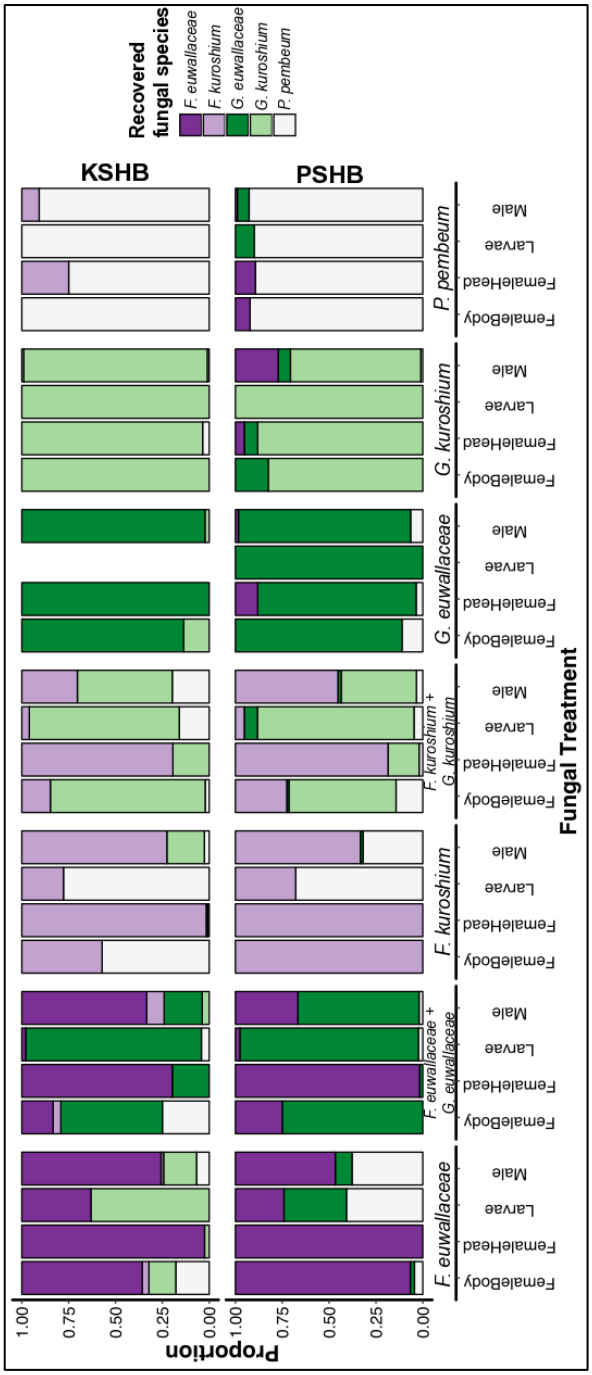


Fig. 3.3 Proportions of each fungal species recovered from all offspring sample types for both beetle species, within each fungal treatment group (no data exists for KSHB larvae on the *G. euwallaceae* treatment).

Table 3.2. Multinomial logistic regression coefficients for fungal species recovery based on beetle species, beetle life stage, and fungal treatment

			Recovered Fungi from Individuals				
Fungal Treatment	**Beetle Type**	**Sample Type**	**Coefficient (%)abc**				
			F. euwallaceae	*G. euwallaceae*	*P. pembeum*	*F. kuroshium*	*G. kuroshium*
Fusarium euwallaceae	PSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	-	-16.03	-15.95	N/A	N/A
		Larvae	-	**3.98**	**3.49**	N/A	N/A
		Males	-	1.94	**2.69**	N/A	N/A
Fusarium euwallaceae	KSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	-	N/A	-17.59	-17.59	**2.17**
		Larvae	-	N/A	-22.14	-22.14	**2.04**
		Males	-	N/A	-1.11	-1.11	0.06
Graphium euwallaceae	PSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	17.07	-	-1.08	N/A	N/A
		Larvae	-0.15	-	16.59	N/A	N/A
		Males	14.99	-	0.57	N/A	N/A
Graphium euwallaceae	KSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	N/A	-	N/A	N/A	-17.29
		Larvae	N/A	-	N/A	N/A	N/A
		Males	N/A	-	N/A	N/A	-1.92
F. euwallaceae + *G. euwallaceae*	PSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	-	**-5.12**	-1.62	N/A	N/A
		Larvae	-	**2.57**	17.69	N/A	N/A
		Males	-	-0.44	14.89	N/A	N/A
F. euwallaceae + *G. euwallaceae*	KSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	-	**-2.58**	-20.08	-18.58	4.89
		Larvae	-	**2.62**	0.29	-15.31	8.17
		Males	-	**-2.36**	-20.06	-0.59	23.07
Paracremonium pembeum	PSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	0.33	-0.01	-	N/A	N/A
		Larvae	-16.45	16.96	-	N/A	N/A
		Males	-2.02	16.46	-	N/A	N/A

Paracremonium pembeum	KSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	N/A	N/A	-	17.14	N/A
		Larvae	N/A	N/A	-	0.01	N/A
		Males	N/A	N/A	-	15.95	N/A
F. kuroshium	PSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	N/A	-0.01	0.01	-	N/A
		Larvae	N/A	-0.59	20.4	-	N/A
		Males	N/A	19.54	18.92	-	N/A
F. kuroshium	KSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	N/A	N/A	**-5.08**	-	12.88
		Larvae	N/A	N/A	0.96	-	-1.16
		Males	N/A	N/A	**-3.68**	-	16.32
G. kuroshium	PSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	15.33	-0.95	17.23	-0.15	-
		Larvae	-0.77	-17.82	0.24	1.24	-
		Males	17.12	-0.82	16.71	16.86	-
G. kuroshium	KSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	N/A	N/A	16.74	0.02	-
		Larvae	N/A	N/A	-0.01	0.01	-
		Males	N/A	N/A	15.53	16.62	-
F. kuroshium + *G. kuroshium*	PSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	N/A	-11.17	-0.73	**2.32**	-
		Larvae	N/A	1.4	**-1.49**	**-2.14**	-
		Males	N/A	0.71	-1.08	**1.04**	-
F. kuroshium + *G. kuroshium*	KSHB	*Female Bodies*	-	-	-	-	-
		Female Heads	N/A	N/A	-12.3	**3.11**	-
		Larvae	N/A	N/A	2.02	-1.3	-
		Males	N/A	N/A	**2.69**	**1.16**	-

a "-" indicates the reference for each multinomial model conducted for each treatment

b "N/A" represents cases where that particular fungal species was not recovered

c Bold numbers indicate Wald-test significance at less than $\alpha = 0.05$

Quantification of fungal genera within different beetle sample types. EMM from Poisson regression models revealed significant differences ($P < 0.05$) in recovery of fungal genera (*Fusarium* spp., *Graphium* spp., and *Paracremonium* sp.) when contrasting by offspring sample type. *Fusarium* spp. recovery was greatest from female heads compared to all other sample types (female bodies, males, and larvae; Figs. S3.1-S3.4). *Graphium* spp. and *P. pembeum* were recovered in the highest concentrations from larvae compared to all other sample types (Figs. S3.1-S3.4). Treatments that contained *F. euwallaceae* or *F. kuroshium* individually or in combination with *Graphium* spp. resulted in recovery of significantly more ($P < 0.05$) *Fusarium* spp. CFUs from the female head, compared to other fungal genera recovered (Fig. S3.1). In treatments without *F. euwallaceae* or *F. kuroshium*, the female heads contained significantly higher CFU counts of the auxiliary fungi that they were treated with (Fig. S3.1). Generally, CFUs recovered from female bodies, males, and larvae were composed of more auxiliary fungal genera, specifically *Graphium* spp., even in the presence of the primary symbionts (Figs. S3.2-S3.4). For example, for both PSHB and KSHB given combination treatments of *F. euwallaceae* + *G. euwallaceae* and *F. kuroshium* + *G. kuroshium,* recovery of *Graphium* spp. was significantly higher ($P < 0.05$) in female bodies than recovery of *Fusarium* spp. or *P. pembeum* (Fig. S3.2). This trend was also seen in larvae and males (Figs. S3.3-S3.4). When given as a treatment, *P. pembeum* was recovered in the highest concentrations compared to other fungal genera across all sample types in both beetle species (Figs. S3.1-S3.4).

Aposymbiotic vs. non-aposymbiotic female reproduction on non-ambrosial *Fusarium* spp. EMM analysis of total offspring counts of aposymbiotic and non-aposymbiotic PSHB females fed symbiotic or non-ambrosial *Fusarium* spp. revealed that reproduction was dramatically reduced on non-ambrosial *Fusarium* spp. Offspring counts from non-aposymbiotic (i.e., untreated) females were significantly higher ($P < 0.05$) in the NTC and primary symbiont (*F. euwallaceae*) treatments compared to the non-ambrosial *F. solani* and *F. oxysporum* treatments (Fig. 3.4). Untreated females reared on the primary symbiont treatment produced the most offspring compared to all other treatments provided. For aposymbiotic virgin females, fewer offspring were observed overall than in untreated females (Fig. 3.5). Aposymbiotic females given the NTC and *F. oxysporum* treatments produced the fewest offspring, while those reared on *F. solani* produced significantly more offspring than those on the NTC and *F. oxysporum* treatments ($P < 0.05$). As with the untreated females, aposymbiotic females given the primary symbiont treatment of *F. euwallaceae* produced the most total offspring ($P < 0.05$).

Quantification of *Fusarium* spp. from aposymbiotic vs. untreated females. In mycangia of untreated females, EMM analysis of CFU counts across all treatments showed that *F. euwallaceae* was present in the highest amounts in both the *F. euwallaceae* and NTC treatments (Fig. 3.6). This was expected, since untreated females given the NTC treatment would have inoculated it with *F. euwallaceae* carried with them from their natal galleries. The non-ambrosial fungi *F. solani* and *F. oxysporum* were

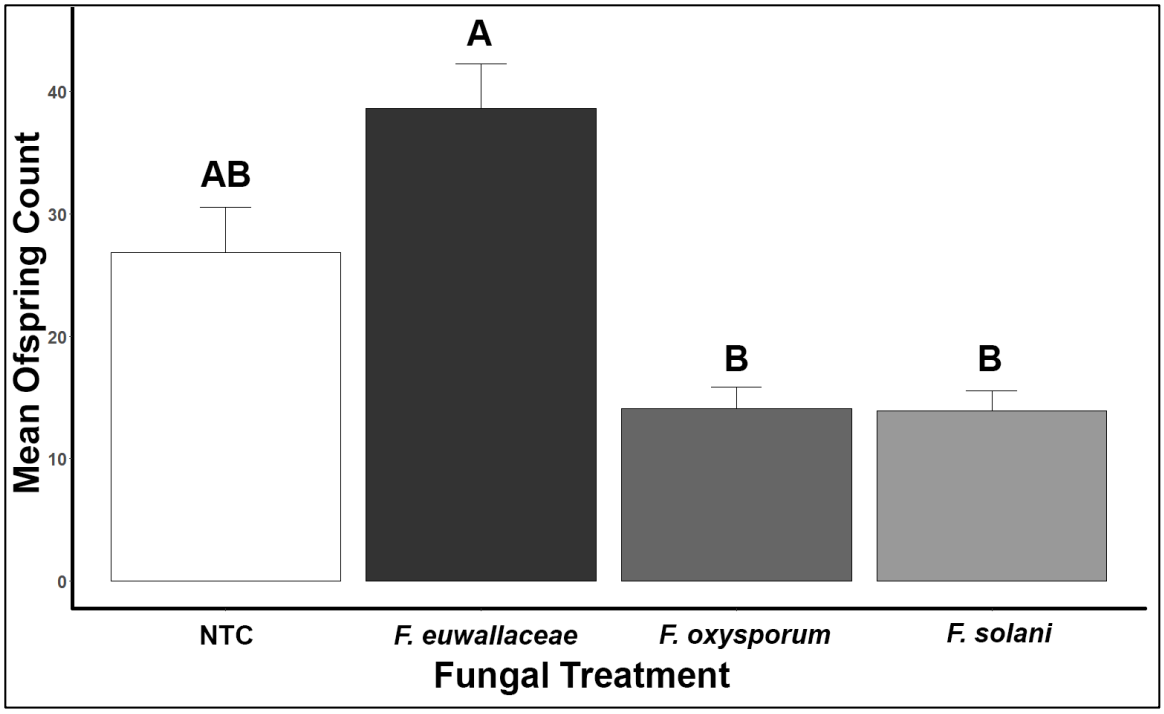


Fig. 3.4 Total offspring counts from all untreated female PSHB, fed symbiotic or non-ambrosial *Fusarium* spp., within each fungal treatment group. Letters represent significant contrasts at $\alpha = 0.05$ using post-hoc EMM from negative binomial regression model.

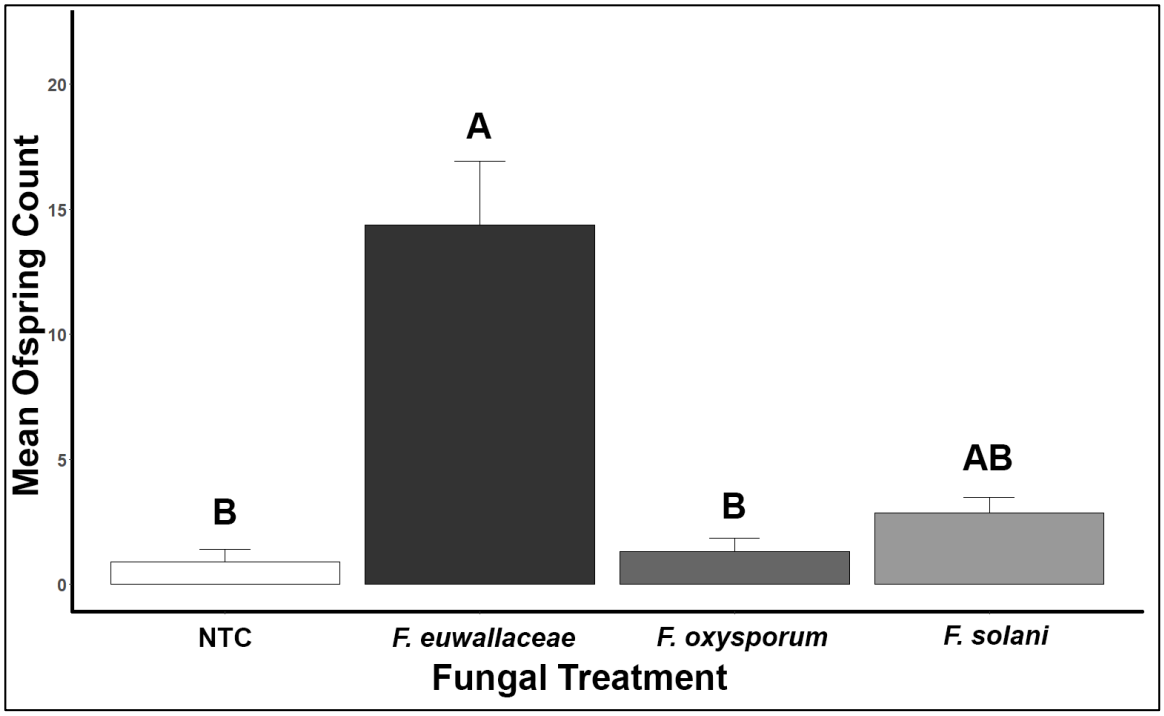


Fig. 3.5 Total offspring counts from all aposymbiotic female PSHB, fed symbiotic or non-ambrosial *Fusarium* spp., within each fungal treatment group. Letters represent significant contrasts at $\alpha = 0.05$ using post-hoc EMM from negative binomial regression model.

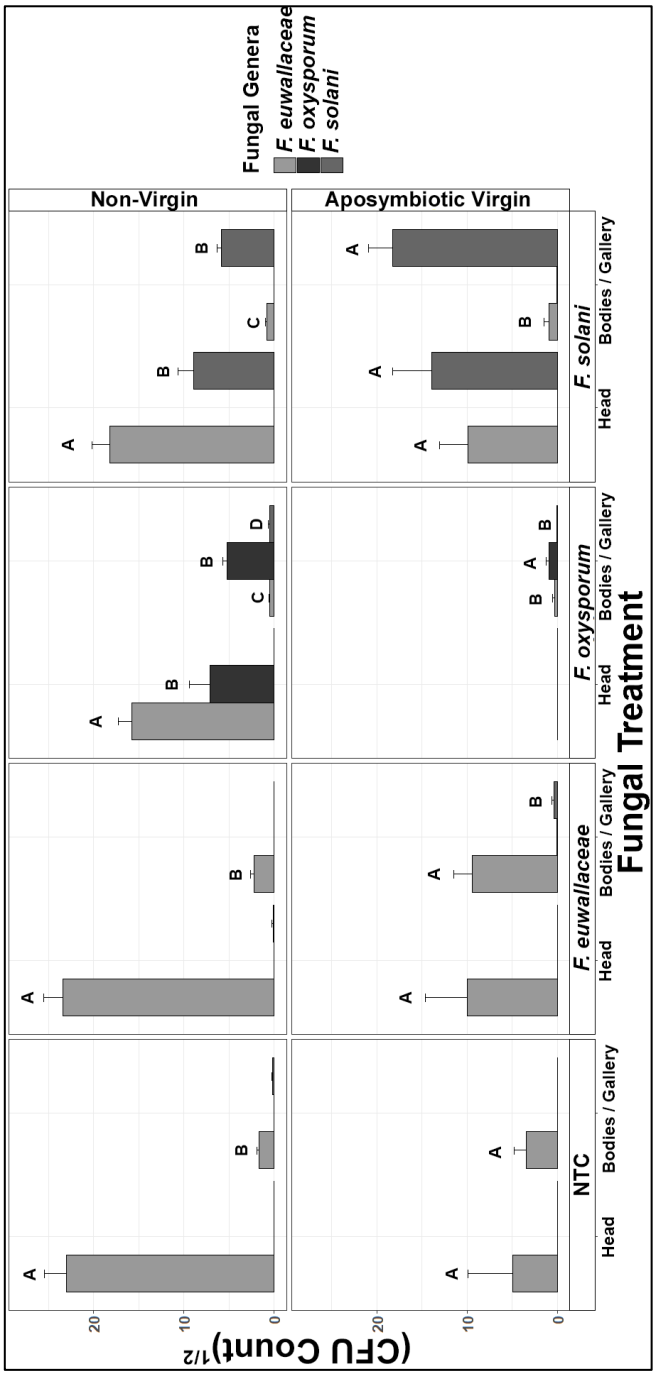


Fig. 3.6 Total CFU counts of each fungal species recovered from the symbiotic vs. non-symbiotic *Fusarium* trial, separated by foundress status. Letters represent significant contrasts within each facet at α = 0.05 using post-hoc EMM from Poisson regression model.

recovered from untreated female mycangia when they were given as treatments, but were still recovered significantly less ($P < 0.05$) than *F. euwallaceae*, regardless of treatment (Fig. 3.6). *F. euwallaceae* was also recovered consistently from aposymbiotic female mycangia across all treatments except *F. oxysporum*, which yielded neither *F. euwallaceae* nor *F. oxysporum* in the mycangia, although both were present in the galleries (Fig. 3.6). When aposymbiotic females were treated with non-ambrosial *F. solani,* recovery of this fungus was greater than that of the primary symbiont *F. euwallaceae* but not significant ($P > 0.05$). Recovery of fungi from other sample types (female bodies, larvae, males, galleries) confirmed that the fungi that were experimentally provided were present in the gallery and were being consumed by offspring (Fig. 3.6).

DISCUSSION

Investigation into the fungal symbiont fidelity of two invasive ambrosia beetles through artificial diet experiments indicate that both species can survive on each other's symbiotic ambrosia fungi. Diets including the primary AFC symbionts *F. euwallaceae* and *F. kuroshium* produced the most total offspring, with no significant differences between PSHB and KSHB when reared on either fungus (Figs. 3.1-3.2). Symbiosis of PSHB with *F. euwallaceae* was previously reported to be specific and exclusive (Freeman et al. 2013a); however, the present study provides evidence for a more promiscuous symbiosis. The high fecundity from treatments that included *F. euwallaceae* or *F. kuroshium* indicate that they are a significant food source that contributes positively

to offspring production in females, which corroborates previous studies that AFC Fusaria are the primary symbionts of *Euwallacea* spp. (Mendel et al. 2012, Kasson et al. 2013, O'Donnell et al. 2015, Na et al. 2018). The addition of auxiliary fungi *G. euwallaceae* and *G. kuroshium* to treatments including AFC Fusaria was not found to significantly increase fecundity, while auxiliary fungal treatments alone produced substantially fewer offspring (Figs. 3.1-3.2), similar to observations on the ambrosia beetle *Xyloborus ferrugineus* and its associated *Graphium* sp. symbiont (Baker and Norris 1968). Auxiliary fungal treatments alone often produced only one generation (all males), while treatments including AFC Fusaria in the diet were more likely to produce a second generation with female offspring. Thus, the auxiliary fungi alone may not be suitable for optimal offspring production, but are shown here to support a cycle of metamorphosis in both PSHB and KSHB.

The removal of female pupae from brood galleries such that eclosion occurs in an aposymbiotic environment has been reported from previous diet experiments (Batra 1966, Baker and Norris 1968), and is advantageous when performing in vitro switching experiments because it frees the mycangia of fungal symbionts. This makes it possible to introduce specific fungi to callow females with empty mycangia in order to study effects on fecundity, fitness, and fungal uptake and consumption when reared on alternative fungi, as shown in the present study. However, in the current study, as well as in previous studies (Batra 1966, Baker and Norris 1968), we were not able to completely dissociate ambrosia beetles from their natural fungal symbionts (Fig. 3.3; Table 3.1). Although the mycangia were verified free of symbionts using our isolation methods (Table 3.1), there

were still cases where symbiotic fungi, mainly the auxiliary fungi *Graphium* spp. or *P. pembeum*, persisted in or on the bodies of females post-eclosion and were recovered from offspring. For instance, offspring were unexpectedly recovered from one KSHB female reared on the negative control treatment *L. citricola* (Figs. 3.1-3.2), but upon further analysis we found that all fungi isolated from these offspring were *G. kuroshium*, indicating that these offspring were feeding upon a symbiont rather than our negative control. Similarly, the bodies (minus the head) of some PSHB females that were fed an alternate diet of *G. kuroshium* retained *G. euwallaceae*, and likewise KSHB females that were fed *G. euwallaceae* retained *G. kuroshium* (Fig. 3.3; Table S3.1). Interestingly, in several female head samples (two PSHB, one KSHB), we recovered both *F. euwallaceae* and *F. kuroshium*. This indicates that the mandibular mycangia of species within the *Euwallacea fornicatus* species complex are capable of simultaneously housing multiple species of primary symbiont, in addition to auxiliary fungi and bacteria. Due to the presence of these other fungi, some of our results may therefore be confounded by symbiont carryover. Selective media has been used to study primary ambrosia fungi such as *Raffalea lauricola*, associated with *Xyloborus glabratus*, using cycloheximide (Fraedrich et al. 2008). Similar approaches could potentially be developed to help control artificial environments and reduce symbiont carryover in ambrosia beetle diet experiments.

Paracremonium pembeum was previously shown not to support larval development when given as a sole food source (Freeman et al. 2015). It was instead proposed to possibly serve as a regulator of contaminants in galleries, similar to

antimicrobial actinomycetes like *Streptomyces* spp. in attine ants (Mueller and Gerardo 2002). However, *P. pembeum* was recovered in high proportions from all samples when given as a treatment (Figs. 3.3, S3.1-S3.4), indicating that it was used extensively as a food source in our experiments. The role of this auxiliary fungus remains unclear. *Graphium euwallaceae* was previously found in significant quantities in the bodies of adult female and larval PSHB, and is suggested to be the dominant food source of immature beetle stages (Freeman et al. 2015). Our results support this hypothesis, and indicate that *Graphium* is also a significant food source for adult beetles; the proportions (Fig. 3.3; Table S3.1) and quantities (Figs. S3.1-S3.4) of *Graphium* spp. in female bodies, larvae, and males were generally higher than what was found in female mycangia in both beetle species. However, the proportions and quantities were largely dependent on the fungal symbiont(s) that individual foundresses were initially treated with. For example, *Graphium* spp. recovery was substantially higher from female bodies, males, and larvae when added in combination with AFC Fusaria compared to the AFC Fusaria treatments alone (Figs. 3.3, S3.2-S3.4). Similarly, when treated with auxiliary *G. euwallaceae, G. kuroshium,* or *P. pembeum* alone*,* greater amounts of each respective fungus were recovered from all sample types, including the female mycangia (Figs. 3.3, S3.1-S3.4; Table S3.1). These observations verify that beetles were feeding mainly on the fungi that were experimentally provided, rather than their own symbionts that might have carried over, but is also significant because auxiliary fungi are generally not dominant in the mycangia; rather, the primary *Fusarium* symbiont is usually dominant in *Euwallacea* spp. (Kasson et al. 2013). The ambrosia beetle mycangium is a dynamic environment, and has

been shown in some species, including PSHB, to become more selective toward the primary symbiont as the adult beetle matures (Kajimura and Hijii 1992, Freeman et al. 2015, Li et al. 2018). Although the vast majority of adult females examined in this study were fully sclerotized (i.e., black in color) and seemingly mature, we cannot rule out the possibility that, if we extracted them prematurely from their gallery, their mycangia were still undergoing changes that might have influenced the fungal community present therein. The fungi that were recovered may not, therefore, be reflective of what foundresses would disperse to new galleries.

Offspring production was significantly reduced in aposymbiotic PSHB that were fed non-ambrosial *Fusarium* spp. (Fig. 3.5). *F. solani* and *F. oxysporum* are well known plant pathogens with a wide host range, mainly as soil-borne pathogens (Ploetz 2006, Coleman 2016) but also as endophytes in some plant species (Evans et al. 2003, Promputtha et al. 2007). The isolates used in this study were not confirmed pathogenic strains, but were used to explore the effects of non-ambrosial *Fusarium* spp. on offspring production and the potential of these fungi to load the mycangia of female PSHB and replace the primary symbiont *F. euwallaceae*. The ability of untreated foundresses to survive and reproduce was suppressed by the presence of non-ambrosial *Fusarium* spp. (Fig. 3.4). Of the two non-ambrosial *Fusarium* spp., PSHB had greater affinity for *F. solani* compared to *F. oxysporum,* particularly in aposymbiotic virgin females (Figs. 3.5-3.6); this is noteworthy because *F. solani* is more closely related to the primary symbiont *F. euwallaceae*, both being from the FSSC. Generally, females retained more symbiotic

F. euwallaceae than non-ambrosial *Fusarium* spp. in their mycangia (Fig. 3.6), which may explain their survival even with pressure from a non-ambrosial fungus.

In a diet tube inoculated with the negative control *L. citricola*, which was not expected to enter the female mycangia, mycelial propagules from a single foundress were recovered from dilution plating the contents of the female head, but no offspring were produced (data not shown). Conversely, in untreated PSHB inoculated with non-ambrosial *F. solani* and *F. oxysporum*, the primary symbiont *F. euwallaceae* dominated the female mycangia even with evidence of the non-symbionts in the galleries and guts of individuals (Fig. 3.6). This may be explained by the previous occupation of the untreated females' mycangia by *F. euwallaceae*, but does not explain how maturing female offspring select for the primary AFC symbiont over the non-ambrosial *Fusarium* spp. present in the galleries. These observations support previous hypotheses regarding mycangial loading: that entry of the spores into the mycangium happens passively as foundresses are exposed to fungi in the galleries, as was suggested by early work on ambrosia beetle mycangia (Batra 1967); then, as the beetle matures, the mycangia become more selective toward certain fungal partners, preferentially retaining those with which it maintains the closest evolutionary relationship (Skelton et al. 2019). The physiological processes governing this selectivity remain elusive, and the legitimacy of these hypotheses will not be proven until tested in other ambrosia beetle systems.

The presence of PSHB and KSHB in California is a great concern to land managers, arborists, and homeowners because of the pests' wide host range. The potential for symbiont sharing adds additional concern for invaded areas where both exist

sympatrically, not least because all fungal symbionts associated with these beetles display some degree of plant pathogenicity and virulence (Eskalen et al. 2012, Freeman et al. 2013b, Lynch et al. 2016, Na et al. 2018). Fungal occupation of the xylem often extends past the point of beetle-spread inoculation and can invade nearby galleries in the same host, providing opportunities for horizontal transmission between beetles in close proximity (Hulcr and Cognato 2010, Carrillo et al. 2014). Recombination in plant pathogenic fungi has been widely studied (Orr-Weaver and Szostak 1985, Milgroom 1996) and can facilitate new genetic combinations, hybridization, and horizontal gene transfer, which can cause clonal emergence of new phytopathogenic lineages (Fisher et al. 2012). Although ambrosia fungi are generally thought to forego sexual reproduction (Beaver 1989), sexual stages have been observed in *Raffaelea vaginata* recovered from ambrosia beetles in South Africa (Musvuugwa et al. 2015), as well as in the mycangial fungus *Ambrosiella cleistominuta*, associated with the ambrosia beetle *Anisandrus maiche* (Mayers et al. 2017). *Fusarium* spp. are known to recombine sexually (Leslie and Summerell 2006) and parasexually (Correll et al. 1987), and this may occur in the AFC Fusaria associated with PSHB and KSHB as well.

The ability of PSHB and KSHB to survive on alternate symbionts has significant implications for California, where no practical approach to managing them exists. Currently, strategies to control the pests are limited (Eatough Jones and Paine 2015, Eatough Jones et al. 2017, Mayorquin et al. 2018) due to their cryptic nature inside woody hosts and the difficulty of delivering effective treatments. These findings highlight the need for a better understanding of the complex insect-microbe interactions that occur

in ambrosia beetle systems, which may help to uncover ways of disrupting and controlling these systems in invaded areas.

Table S3.1. Multinomial logistic regression coefficients comparing fungal species recovery, based on fungal treatment and beetle species

		Recovered fungi				
Fungal treatment	**Beetle species**	**Coefficient (%)abc**				
		F. euwallaceae	*G. euwallaceae*	*P. pembeum*	*F. kuroshium*	*G. kuroshium*
Fusarium euwallaceae	KSHB	-	-	-	-	-
	PSHB	-	18.49	**1.34**	-18.11	-18.46
Graphium euwallaceae	KSHB	-	-	-	-	-
	PSHB	16.57	-	16.6	N/A	-17.52
F. euwallaceae + *G. euwallaceae*	KSHB	-	-	-	-	-
	PSHB	-	0.31	**-1.17**	-15.98	-15.52
Paracremonium pembeum	KSHB	-	-	-	-	-
	PSHB	17.98	17.24	-	-18.22	N/A
F. kuroshium	KSHB	-	-	-	-	-
	PSHB	N/A	17.19	0.54	-	-16.76
G. kuroshium	KSHB	-	-	-	-	-
	PSHB	17.29	17.38	-0.71	-16.56	-
F. kuroshium + *G. kuroshium*	KSHB	-	-	-	-	-
	PSHB	N/A	15.22	-0.4	**0.57**	-

a "-" indicates the reference for the multinomial model conducted for that treatment
b "N/A" indicates that particular fungal species was not recovered
c Bold numbers indicate Wald-test significance at $\alpha = 0.05$

Table S3.2. Multinomial logistic regression coefficients for each recovered fungal species, based on beetle sample type and fungal treatment

		Recovered Fungi from Individuals				
Fungal Treatment	**Sample Type**	**Coefficient (%)abc**				
		F. euwallaceae	*G. euwallaceae*	*P. pembeum*	*F. kuroshium*	*G. kuroshium*
Fusarium euwallaceae	Female Bodies	-	-	-	-	-
	Female Heads	-	-17.15	-18.62	-19.63	**-1.97**
	Larvae	-	**3.69**	**1.93**	-23.13	**2.5**
	Males	-	1.74	**1.23**	-0.87	0.31
Graphium euwallaceae	Female Bodies	-	-	-	-	-
	Female Heads	17.36	-	-1.08	N/A	-17.18
	Larvae	-0.52	-	-17.25	N/A	-1.59
	Males	15.29	-	-0.57	N/A	-1.91
F. euwallaceae + *G. euwallaceae*	Female Bodies	-	-	-	-	-
	Female Heads	-	**-3.72**	-18.83	-16.84	-0.07
	Larvae	-	**-2.61**	1.13	-13.75	3.01
	Males	-	**-1.04**	**-2.61**	0.12	17.04
Paracremonium pembeum	Female Bodies	-	-	-	-	-
	Female Heads	0.34	-0.18	-	17.65	N/A
	Larvae	-18.34	18.21	-	0.67	N/A
	Males	-2.02	17.71	-	16.47	N/A
F. kuroshium	Female Bodies	-	-	-	-	-
	Female Heads	N/A	0.48	**-3.67**	-	13.31
	Larvae	N/A	-5.4	**2.52**	-	-5.45
	Males	N/A	18.17	0.13	-	16.85
G. kuroshium	Female Bodies	-	-	-	-	-
	Female Heads	15.33	-0.95	17.23	-0.15	-
	Larvae	-0.77	-17.82	0.24	1.24	-
	Males	17.12	-0.82	16.71	16.86	-
F. kuroshium + *G. kuroshium*	Female Bodies	-	-	-	-	-
	Female Heads	N/A	-13.27	-0.85	**2.65**	-
	Larvae	N/A	1.55	-0.34	**-1.86**	-
	Males	N/A	0.82	0.37	**1.1**	-

a "-" indicates the reference for the multinomial model conducted for that treatment
b "N/A" indicates that particular fungal species was not recovered
c Bold numbers indicate Wald-test significance at $\alpha = 0.05$

Table S3.3. Proportion table representing recovered fungal species from each beetle species and life stage by fungal treatment

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

a "N/A" represents cases where individuals were not sampled
b "-" indicates that particular fungus was not recovered

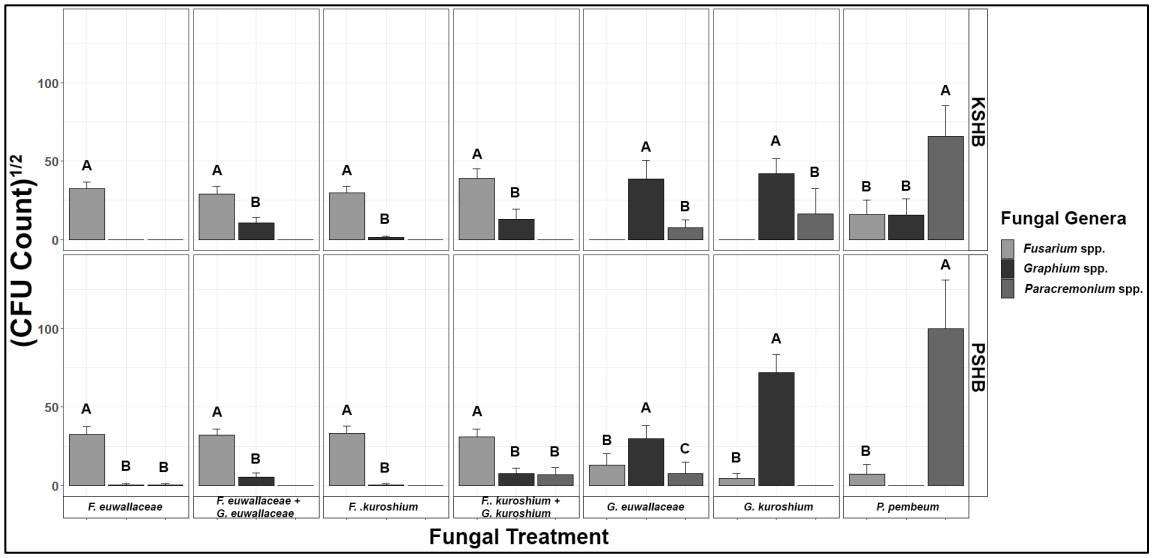


Fig. S3.1 CFU counts of each fungal genus recovered from female heads, separated by beetle species and fungal treatment. Letters represent significant contrasts within each facet at α = 0.05 using post-hoc EMM from Poisson regression model.

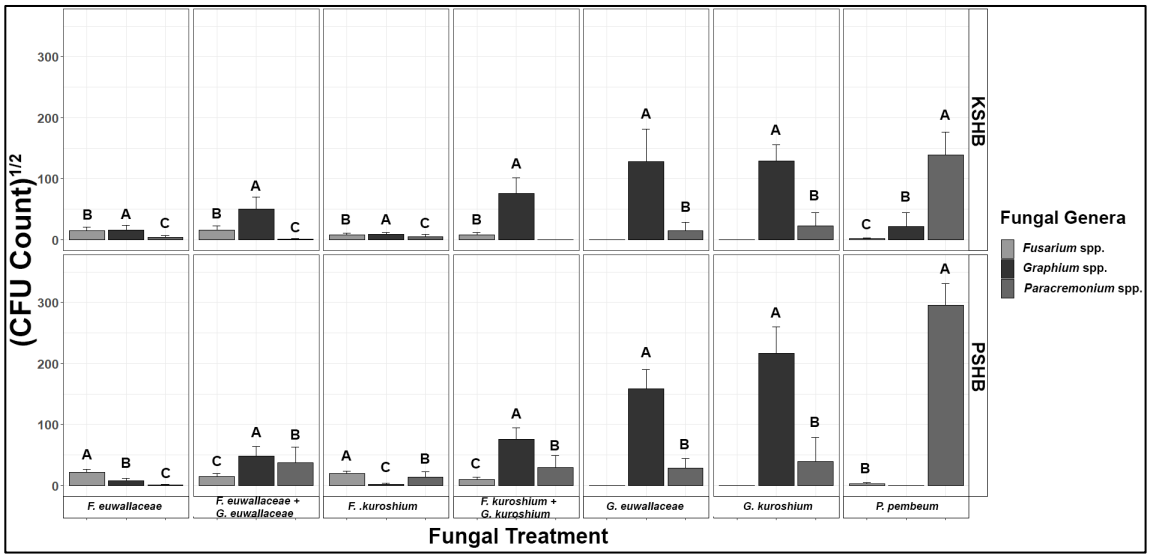


Fig. S3.2 CFU counts of each fungal genus recovered from female bodies, separated by beetle species and fungal treatment. Letters represent significant contrasts within each facet at α = 0.05 using post-hoc EMM from Poisson regression model.

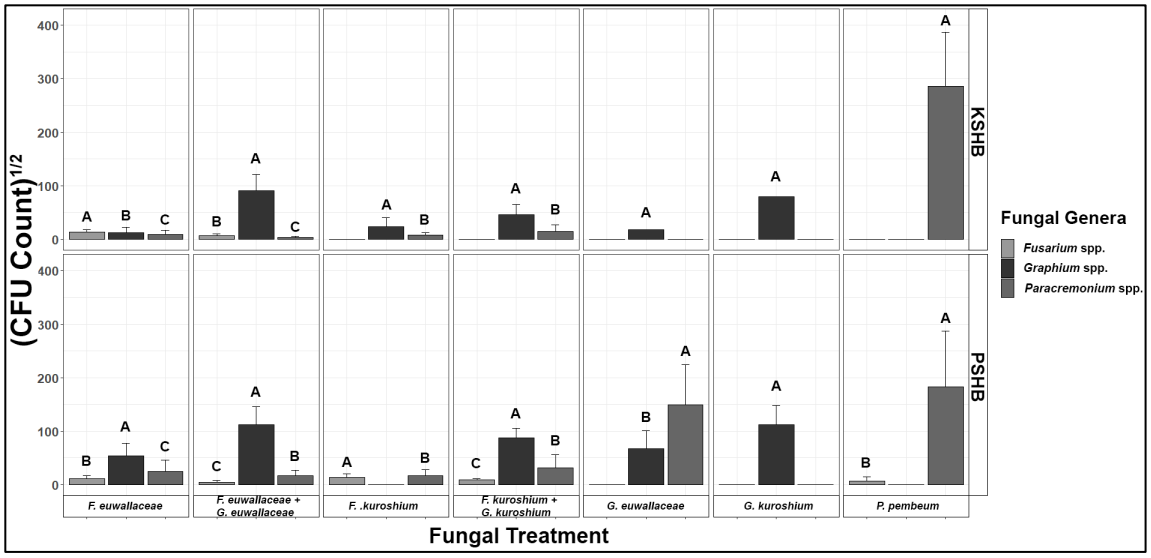


Fig. S3.3 CFU counts of each fungal genus recovered from larvae, separated by beetle species and fungal treatment. Letters represent significant contrasts within each facet at α = 0.05 using post-hoc EMM from Poisson regression model.

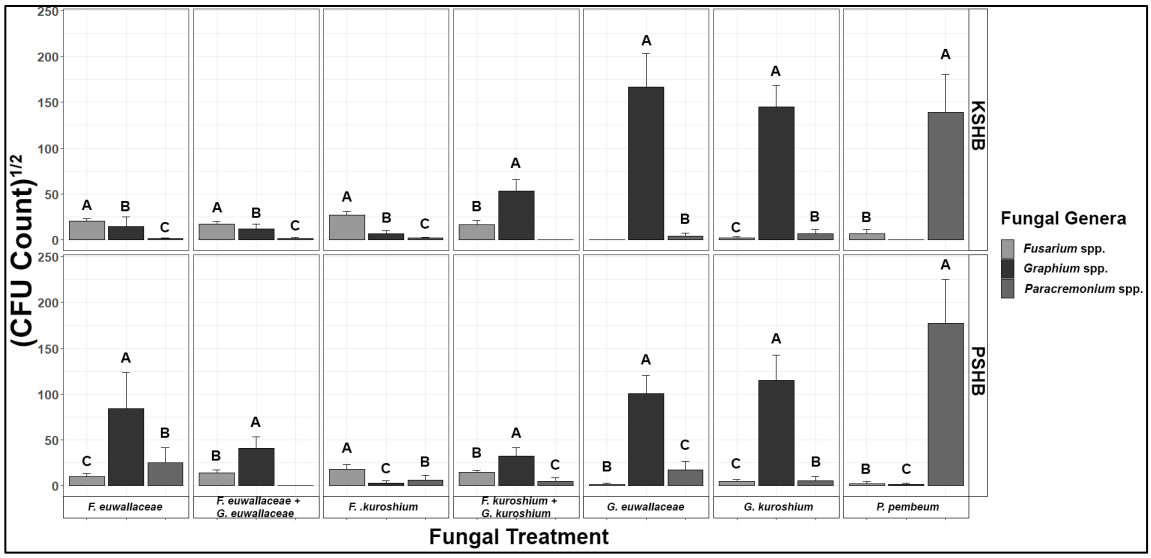


Fig. S3.4 CFU counts of each fungal genus recovered from males, separated by beetle species and fungal treatment. Letters represent significant contrasts within each facet at α = 0.05 using post-hoc EMM from Poisson regression model.

Chapter IV. Population genetics of invasive populations of polyphagous and Kuroshio shot hole borers in California.

ABSTRACT

Sufficient knowledge of genetic structure of invasive populations is crucial for formulating efficient management plans. The polyphagous and Kuroshio shot hole borers, two members of a cryptic ambrosia beetle species complex, are recently invasive in southern California and are potentially devastating pests of over 60 species of local hardwood trees. Populations of shot hole borers are expected to be extremely homozygous due to the fact that siblings usually mate with each other inside the natal gallery, but their proclivity for outbreeding has never been explicitly tested. Knowledge of their genetic diversity in invaded areas is lacking and would inform management, in addition to revealing mating structure. Here, we give the first report of population genetic structure of shot hole borers in California, elucidated from double digest restriction enzyme-associated DNA sequencing. We find lower levels of homozygosity and sib-mating in invasive shot hole borer populations than expected given a strictly inbreeding lifestyle. We also see signatures of geographical population structuring in certain populations of California shot hole borers.

INTRODUCTION

Invasive species are a global concern as threats to agriculture, native species, and human health. Management strategies for invasive species rely on rapid and accurate species identification, which is complicated by cryptic species complexes, in which genetically divergent species are morphologically indistinguishable (Bickford et al. 2007). Genetic studies of invasive populations are useful in detecting cryptic species, and can also help predict and track population dispersal as they spread across an invaded area (Sakai et al. 2001). They are also crucial for assessing genetic diversity, an important factor to consider when formulating invasive species management plans.

For non-model organisms or organisms for which no genomic data is available, reduced representation genome sequencing approaches have proven useful in accurately describing *de novo* genomic diversity (Davey et al. 2011). In particular, restriction enzyme-associated DNA sequencing (RADSeq) methods are becoming increasingly popular tools to characterize genetic diversity of non-model populations (Baird et al. 2008). RADSeq methods use restriction enzymes, which cleave DNA at specific cutsites across the genome, to digest the DNA of samples from populations of interest. This results in the sequencing of homologous genomic segments (loci) that are shared between all individuals sampled. These loci and any variable regions therein, such as single nucleotide polymorphisms (SNPs), can then be compared within and between populations to assess levels of diversity and admixture. With the advent of double digest RADSeq (ddRADSeq; Peterson et al. 2012), which uses two restriction enzymes simultaneously, users can optimize the workflow for their specific study organism by

dictating roughly how many loci will be generated based on whether the chosen cutsites are rare or common across the genome. This method is also more robust than traditional RADSeq when working with organisms that lack a reference genome, since it only retains loci that have cutsites from both restriction enzymes (i.e., one at either end). Paired with strict size selection, this ensures that only truly homologous loci are sequenced (Peterson et al. 2012).

Euwallacea fornicatus Eichhoff, commonly called the polyphagous shot hole borer (PSHB), and *E. kuroshio* Gomez & Hulcr, commonly called the Kuroshio shot hole borer (KSHB), are two invasive species of concern in Southern California (Eskalen et al. 2012, Gomez et al. 2018, Smith et al. 2019). Both are ambrosia beetles (Coleoptera: Curculionidae: Scolytinae), minute wood-boring beetles that associate with mutualistic "ambrosia" fungi which they farm as a food source (Beaver 1989). Unlike most ambrosia beetles, PSHB and KSHB attack live, apparently healthy trees, and their ambrosia fungi are at least mildly phytopathogenic (Lynch et al. 2016, Na et al. 2018). Boring damage from beetle activity paired with fungal invasion into the host xylem causes the plant disease Fusarium dieback, which affects over 60 tree species in natural, urban, and agricultural landscapes in Southern California (Eskalen et al. 2013).

PSHB and KSHB are both members of the cryptic *Euwallacea fornicatus* species complex, whose members are invasive worldwide (Cooperband et al. 2016, Stouthamer et al. 2017, Gomez et al. 2018, Smith et al. 2019). *E. perbrevis* Schedl, the tea shot hole borer (TSHB), has long been a serious pest of tea in Southeast Asia (Gadd 1941, Walgama and Pallemulla 2005) and is invasive in the United States in Florida and Hawaii

(Stouthamer et al. 2017). PSHB was first detected in California in 2006 (Rabaglia et al. 2006) and identified as TSHB, until DNA sequencing revealed substantial genetic divergence between them (Eskalen et al. 2013). Likewise, KSHB was discovered in California in 2013 and was mistaken for a second invasion of PSHB until molecularly identified. Sequencing of appropriate gene regions, such as the mitochondrial cytochrome *c* oxidase subunit I (COI) "barcoding" region (Hebert et al. 2003), remains the only reliable method of distinguishing members of this species complex (Stouthamer et al. 2017, Gomez et al. 2018, Smith et al. 2019).

The *Euwallacea fornicatus* species complex belongs to the ambrosia beetle tribe Xyleborini, all members of which are haplodiploid, inbreeding species (Normark et al. 1999). Haploid males mate with their diploid sisters within their natal gallery, and fertilized females then disperse to form brood galleries of their own (Kirkendall 1983, Cooperband et al. 2016). Inbreeding leads to a reduction in genetic variation over evolutionary time, so populations of xyleborine beetles are expected to be extremely homozygous. This reduction in genetic diversity is likely to be even more extreme in recently invaded areas due to founder effects. However, signatures of outbreeding have been documented in other ambrosia beetles under certain environmental conditions (Holzman et al. 2009) or in certain populations of select taxa (Peer and Taborsky 2005, Keller et al. 2011, Storer et al. 2017), although it has not been explored extensively. The consequences of outbreeding in xyleborine beetles are thus unknown: outbreeding may introduce sufficient genetic diversity to allow them to exploit their environment more efficiently, becoming more invasive; conversely, it could result in outbreeding

depression, a decrease in fitness that occurs when parents are too genetically dissimilar, due to the intermixing of genetic material that does not share a coevolved history (Lynch 1991). In California, the invasive PSHB population is comprised of two distinct COI haplotypes, here designated H33 and H35, which differ by a single nucleotide; Californian KSHB are represented by a single COI haplotype (Rugman-Jones and Stouthamer 2016, Stouthamer et al. 2017). Additionally, we have discovered heteroplasmic PSHB in California that contain both COI haplotypes within an individual (Dodge et al., in prep). PSHB and KSHB have been shown to hybridize with low success in laboratory crossing experiments (Cooperband et al. 2017), but it is unknown whether they are interbreeding in the field.

Currently, there is little knowledge of the genetic structure of invasive populations of PSHB and KSHB, but the hidden diversity of these cryptic pests necessitates further investigation of the genetic diversity in this group. We used ddRADSeq to perform genetic analysis of invasive populations of PSHB and KSHB in California. Our objectives were to i) gain a baseline knowledge of population genetic diversity and structure in these populations; and ii) determine the extent of outbreeding in these populations. We used SNP data generated from ddRADSeq to compare observed and expected levels of heterozygosity within and between populations, and used these to calculate F-statistics (Weir and Cockerham 1984), measure departure from Hardy-Weinberg equilibrium (HWE), and assess genetic diversity and admixture. Under complete inbreeding, we expect that all loci are highly homozygous and significantly departed from HWE.

MATERIALS AND METHODS

Specimen Collection, Genotyping, and DNA Extraction. Adult female PSHB and KSHB were collected from various field experiments and monitoring stations around Southern California (Fig. 4.1, Table 4.1) between the years 2014-2018 and stored in 95% ethanol at -20°C. PSHB were collected from the following localities: La Habra Heights (LHH); the Huntington Library, Art Collections, and Botanical Gardens, Pasadena (PAS); Prado Dam and Santa Ana River, Corona (COR); Irvine Regional Park, Orange (IRP); and various sites in Ventura County (VEN). KSHB were collected from the following localities: Escondido (ESC); San Diego Zoo, San Diego (SND); and Irvine Regional Park, Orange (IRPK), where it is sympatric with PSHB.

Specimens were genotyped to species using a previously described quantitative PCR assay paired with high-resolution melt (HRM) analysis (Rugman-Jones and Stouthamer 2016). PSHB specimens were haplotyped using a custom locked nucleic acid (LNA)-probe based assay (Dodge et al., in prep.). DNA was extracted from a total of 211 PSHB and 85 KSHB, using a QIAGEN DNeasy Blood & Tissue Kit (Venlo, the Netherlands). Prior to DNA extraction, the heads of the specimens were removed from the rest of the body using a sterile knife to prevent carryover and amplification of non-target DNA from the mandibular mycangia (fungus storage organs) of female beetles. DNA was quantified using an Invitrogen™ Qubit 2.0 Fluorescence Reader (Thermo Fisher Scientific, Waltham, MA, USA) at the University of California, Riverside (UCR) Institute for Integrative Genome Biology.

DNA Library Preparation and Sequencing. DNA library preparation for ddRADSeq was performed following the methods of Brelsford et al. (2016), using custom barcodes 4-7 bp in length for dual-indexing. Briefly, genomic DNA was digested using the restriction enzymes *SbfI* and *MseI*, and resulting fragments were subjected to two rounds of PCR amplification. An intial cleaning and size selection step was performed using homemade Ampure XP in a 0.8:1 ratio of Ampure to sample, to remove short fragments up to 300 bp in length. A second size selection step was performed using Pippin Prep (Sage Science, Beverly, MA, USA), which retained only fragments between 300-600 bp. The final library of 279 samples (68 KSHB, 211 PSHB) was sequenced on a single Illumina HiSeq4000 (Illumina Inc., San Diego, CA, USA) lane at the University of California, Berkeley QB3 Genomic Sequencing Laboratory (Berkeley, CA, USA).

Read Processing and Analysis. Raw 100 bp single-end reads were demultiplexed, trimmed to a uniform length of 93 bp, and quality filtered using the *process_radtags* program in STACKS v2 (Catchen et al. 2013). Currently, no reference genome exists for any *Euwallacea* species, so resulting FASTA files were analyzed using the *denovo_map* program in STACKS. This function first aligns reads across samples into matching "stacks" of homologous loci (i.e., putative alleles), then creates a catalog of these alleles to which each sample is back-referenced.

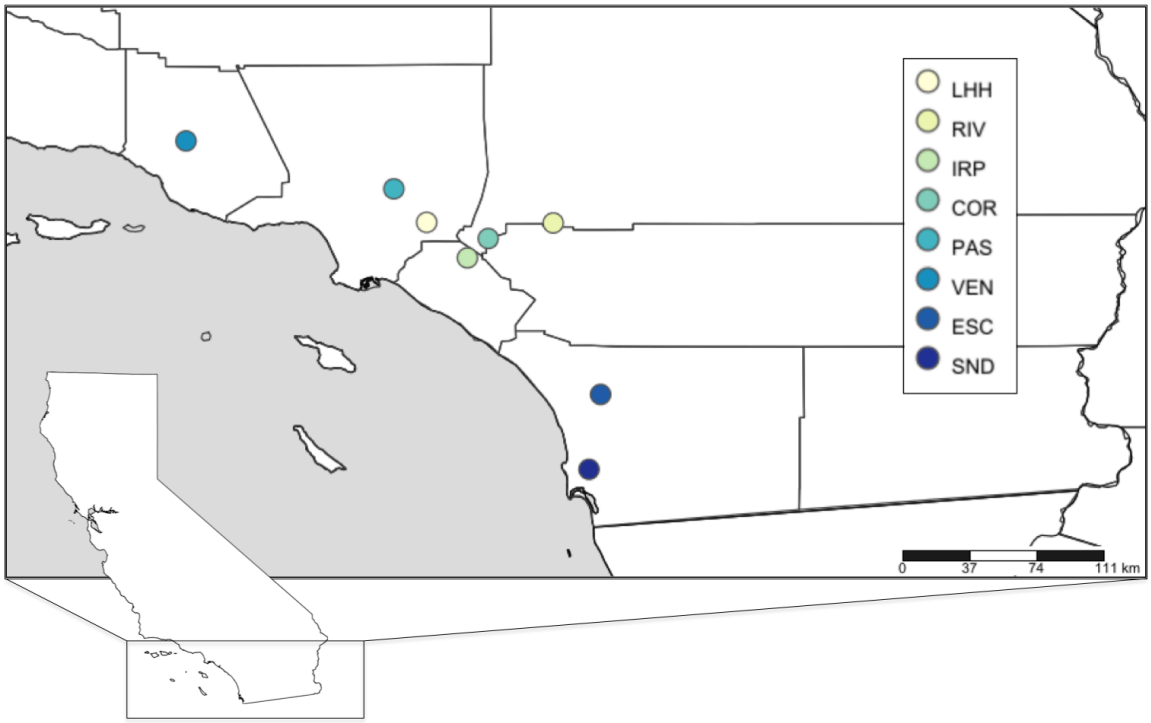


Fig. 4.1 Map of sampling localities across Southern California. Inset shows the sampled region within the state of California. Legend abbreviations: LHH = La Habra Heights; RIV = Riverside; IRP = Irvine Regional Park; COR = Corona; PAS = Pasadena; VEN = Ventura; ESC = Escondido; SND = San Diego. Both species were collected from IRP.

Table 4.1 Number of PSHB and KSHB specimens (N) sequenced from each locality.

PSHB	**N**	**KSHB**	**N**
La Habra Heights (LHH)	50	Escondido (ESC)	34
Riverside (RIV)	17	San Diego (SND)	24
Irvine Regional Park (IRP)	30	Irvine Regional Park (IRPK)	10
Corona (COR)	47	**Total**	**68**
Pasadena (PAS)	38		
Ventura (VEN)	28		
Total	**210**		

Using this catalog, the function then calls SNPs and performs population-level analyses using the *populations* program. Following parameter testing set forth by Paris et al. (2017), we ran *denovo_map* with the following core parameter settings: *m*, the minimum number of raw reads within an individual required to form a stack, was set to 3; *M*, the number of mismatches allowed between stacks to merge them into a single polymorphic locus within an individual, was set to 2; and *n*, the number of mismatches allowed between stacks from different individuals during catalog construction, was set to 3. Because of the subjectivity of performing comparative analyses on populations with unknown genetic structure, we performed both a "strict" and a "relaxed" run of the *populations* program. Specifically, the parameter *r*, which indicates the minimum proportion of individuals in a population that must possess a given locus for that locus to be included in the analysis, was set to 0.50 for the strict analysis and 0.15 for the relaxed analysis. This was done to compare the number of SNPs and polymorphic loci recovered and give insight into the genetic diversity present at both more conserved (strict) and more variable (relaxed) levels. The parameter *p*, the minimum number of populations a locus must be present in, was set to 1 to help illustrate the genetic diversity present at each sampled location. The above analyses were performed separately for PSHB and KSHB, as well as on the total combined dataset, using specimen locality information (Table 4.1) to designate populations. For PSHB, we ran additional analyses on each locality separately and designated population by haplotype, to determine the level of admixture occurring between haplotypes within a geographic population.

After calling SNPs, STACKS outputs results in a variety of file formats accepted by other population genetics software programs. We used VCFTOOLS (Danecek et al. 2011) to calculate the observed heterozygosity (H_{obs}) and expected heterozygosity (H_{exp}) at each variant site, using the *hardy* function. From these values we calculated the inbreeding coefficient, F, as a measure of departure from HWE (Wright 1950). F is calculated using the following equation:

$$F = 1 - \frac{H_{obs}}{H_{exp}}$$

F ranges from 0 to 1; at F = 0, H_{obs} is equal to H_{exp} and genotype frequencies are thus in HWE. At F = 1 there are no heterozygotes, indicative of continuous inbreeding. Using F, we calculated the probability of sib-mating, s (Greeff 2002), at each site:

$$s = \frac{4F}{(1+3F)}$$

Hierarchical F-statistics, specifically F_{IS} and F_{ST}, were calculated in R v3.4.3 (R Core Team 2017) using the package 'hierfstat' (Goudet 2005). F_{IS} is a measure of how inbred the individuals within a population are, and ranges from -1 (complete outbreeding) to 1 (complete inbreeding). F_{ST}, also known as the fixation index, is a measure of how admixed populations are with each other, and is reported here as pairwise values between populations. F_{ST} ranges from 0 (all shared genes) to 1 (no shared genes).

We also used discriminant analysis of principal components (DAPC; Jombart et al. 2010) to identify and describe genetic clustering between populations, using the 'adegenet' package in R (Jombart and Ahmed 2011). DAPC maximizes variance between groups while minimizing variance within groups, and is thus useful for visualizing the

underlying structure of groups that are highly genetically similar. Unlike other programs for describing multilocus genotype data, such as STRUCTURE (Pritchard et al. 2000), DAPC is non-parametric and does not rely on prior assumptions or the application of specific evolutionary models to the data. The first step in DAPC is to perform principal component analysis (PCA), which reduces the dimensions of the data (Liu and Zhao 2006, Lee et al. 2009). Then, comparable to STRUCTURE, DAPC employs *k*-means clustering on the PCA-transformed data, resulting in different likelihoods for different numbers of clusters (i.e., populations). Clustering outcomes are compared using Bayesian Information Criterion (BIC) to determine the most likely number of populations (K). Finally, DAPC provides posterior membership probabilities for each individual to each population, detailing genetic structure and potential admixture between populations.

RESULTS

Sequencing and Catalog Construction. After demultiplexing and quality filtering, 221,202,515 total reads were retained with an average of 249,962 reads and 57.6x coverage per individual. One PSHB sample was discarded due to low sequencing quality, so in total 68 KSHB and 210 PSHB samples were used in final analyses (Table 4.1).

The number of catalogued loci and SNPs recovered from each analysis can be found in Table 4.2. Only biallelic SNPs were retained. Much of the variation within populations was lost in strict analyses, which required loci to be present in at least 50% of individuals in a population (Table 4.2, Fig. S4.1), compared to relaxed analyses.

Departure from Hardy-Weinburg Equilibrium. The vast majority of SNPs recovered from relaxed analyses were completely homozygous (F = 1; Figs. S4.2-4.3), indicative of extremely high levels of inbreeding. The average probability of sib-mating, s, across all variant sites recovered from relaxed analyses was 0.94 and 0.90 for PSHB and KSHB, respectively (Figs. 4.2-4.3). However, many of the SNPs retained in strict analyses were heterozygous, and the average sib-mating probability was 0.74 and 0.61 for PSHB and KSHB, respectively (Figs 4.2-3). This indicates that the more conserved loci (i.e., present within a majority of individuals) within a population were also the most divergent. Overall, values of s and F were lower (i.e., closer to HWE) in loci recovered from KSHB compared to PSHB (Figs. 4.2-3). Most populations sampled were shown to have extremely high F_{IS} values (> 0.90), with little difference between strict and relaxed analyses (Table 4.3), providing further evidence of high levels of inbreeding. However, the ESC and SND populations of KSHB and VEN population of PSHB were found to exhibit slightly higher levels of outbreeding (Table 4.3).

Table 4.2 Number of loci and SNPs recovered from each *denovo_map* analysis.

Analysis	**# loci**	**# SNPs**
Combined relaxed	9,300	27,900
Combined strict	1,049	3,147
PSHB relaxed	7,076	21,228
PSHB strict	217	651
KSHB relaxed	2,595	7,785
KSHB strict	438	1,314

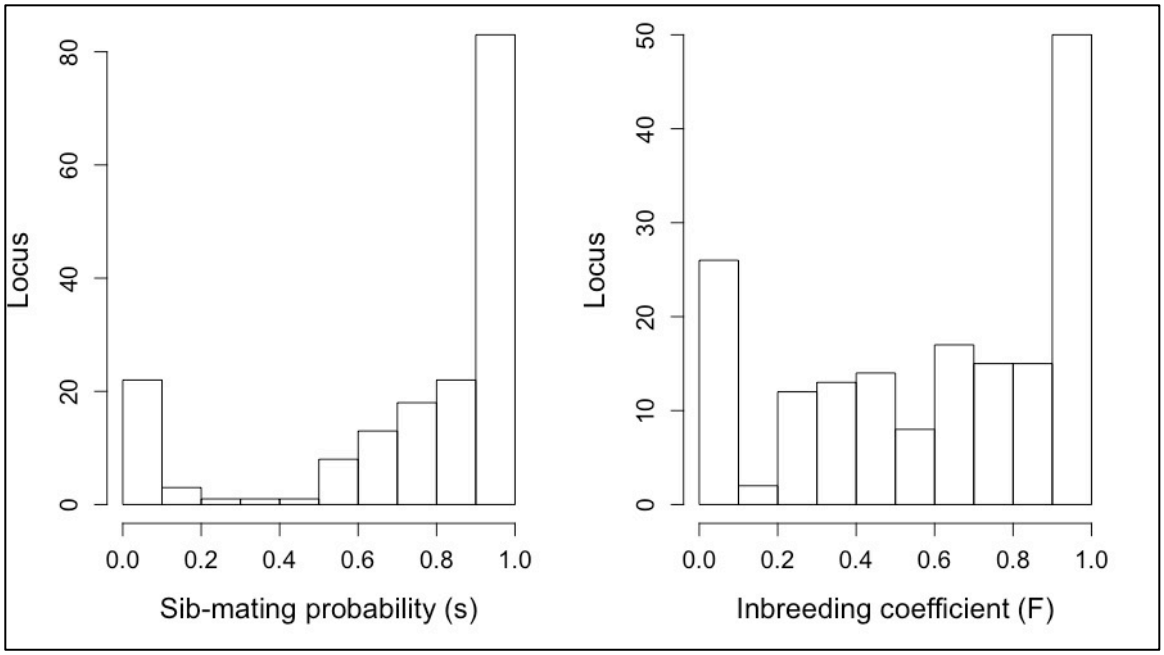


Fig. 4.2 Histogram of sib-mating probabilities (s) and inbreeding coefficients (F) for all recovered loci from PSHB-only analysis (strict), with the number of loci on the y-axis. A value of 1 indicates that a locus is completely homozygous, whereas a value of 0 indicates that the locus is in HWE.

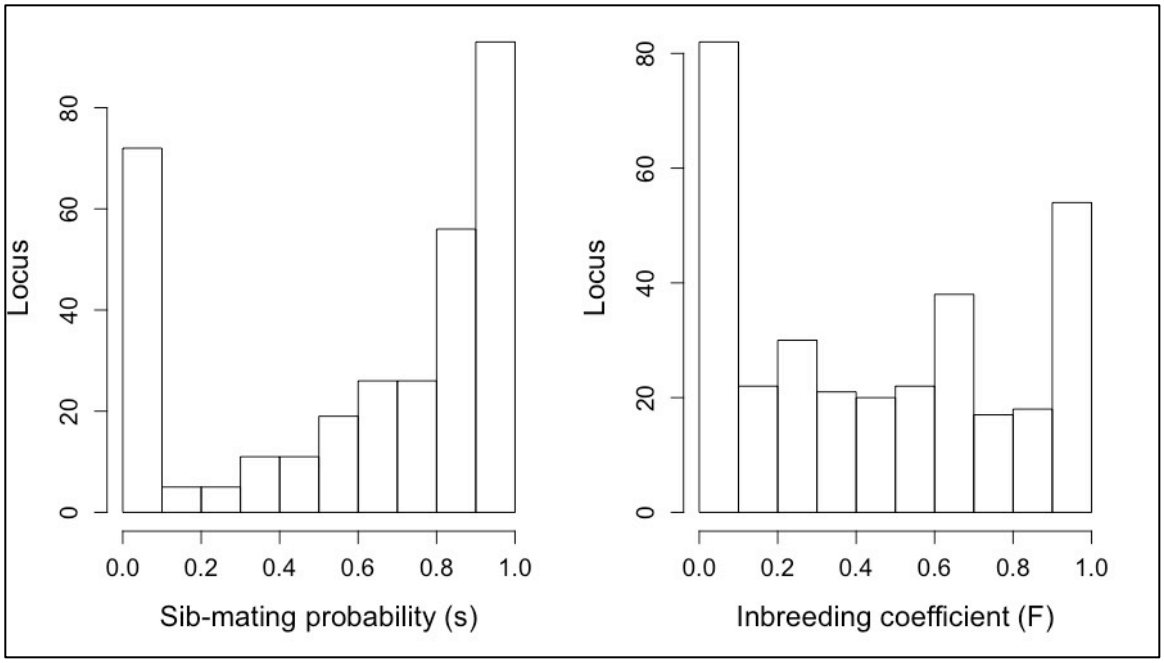


Fig. 4.3 Histogram of sib-mating probabilities (s) and inbreeding coefficients (F) for all recovered loci from KSHB-only analysis (strict), with the number of loci on the y-axis. A value of 1 indicates that a locus is completely homozygous, whereas a value of 0 indicates that the locus is in HWE.

Table 4.3 F_{IS} values for all populations.

Population	LHH	RIV	IRP	COR	PAS	VEN	ESC	SND	IRPK
F_{IS} (relaxed)	0.917	0.965	0.958	0.906	0.929	0.888	0.770	0.841	0.923
F_{IS} (strict)	0.925	0.965	0.963	0.904	0.935	0.878	0.758	0.847	0.926

Genetic Diversity and Admixture. Significantly more SNPs were recovered from the COR population of PSHB than any other population in the relaxed analysis; however, the strict analysis recovered roughly similar numbers of SNPs for all populations considered (Fig. S4.1). F_{ST} values, while slightly higher between species, were relatively low between all pairs of populations, indicating low levels of divergence between populations (Table 4.4). DAPC revealed maximal discrimination between populations, making it possible to discern population structure (Figs. 4.4-4.6). When considering the relaxed analysis, DAPC posterior probability assignment revealed that most PSHB individuals from LHH, RIV, and IRP had a relatively low probability of being matched to their original population, indicating that there is substantial gene flow between these populations and they are not genetically distinguishable (Figs. S4.8). However, many PSHB individuals from COR,

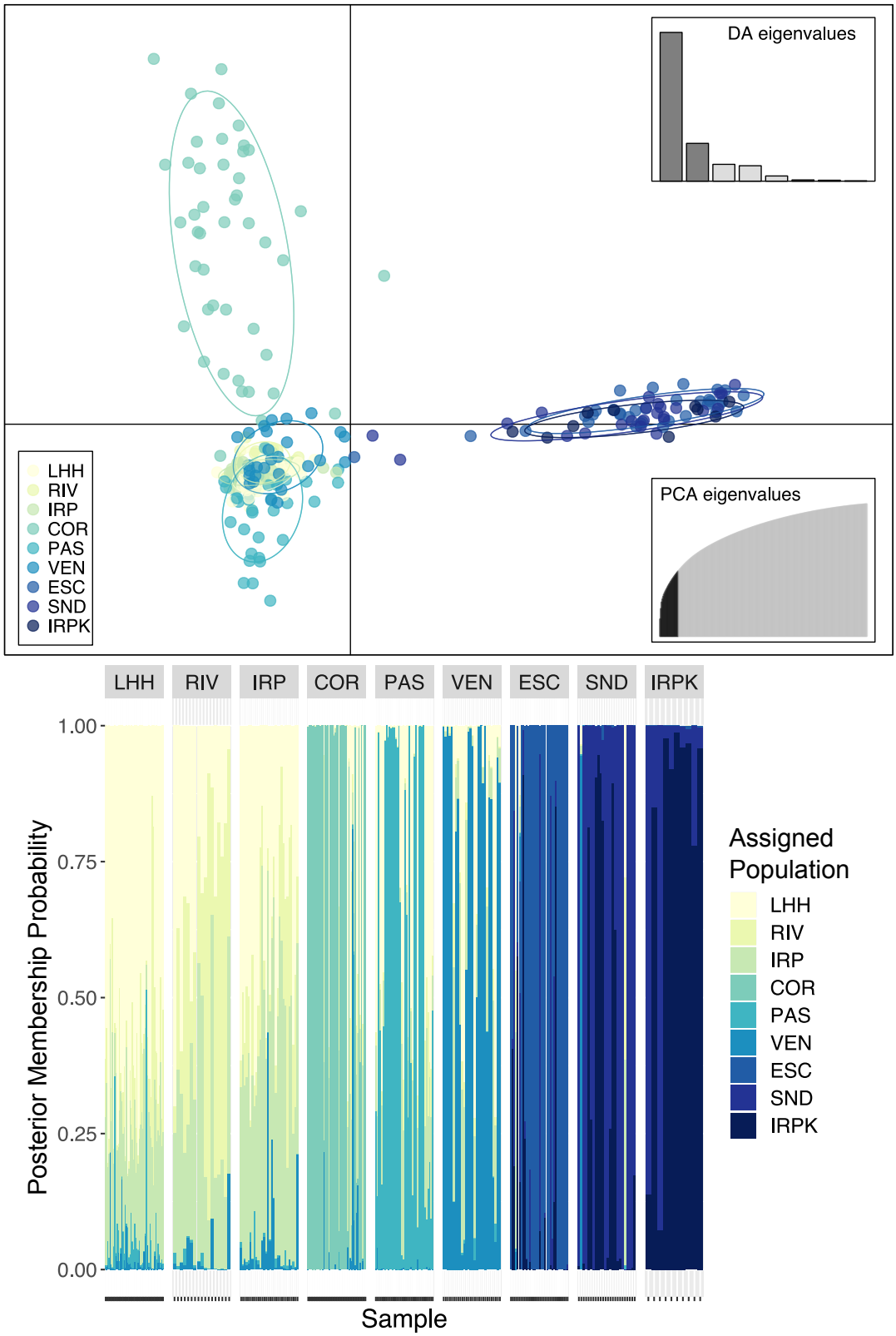
Table 4.4 Pairwise Nei's F_{ST} between populations across all loci (strict analysis).

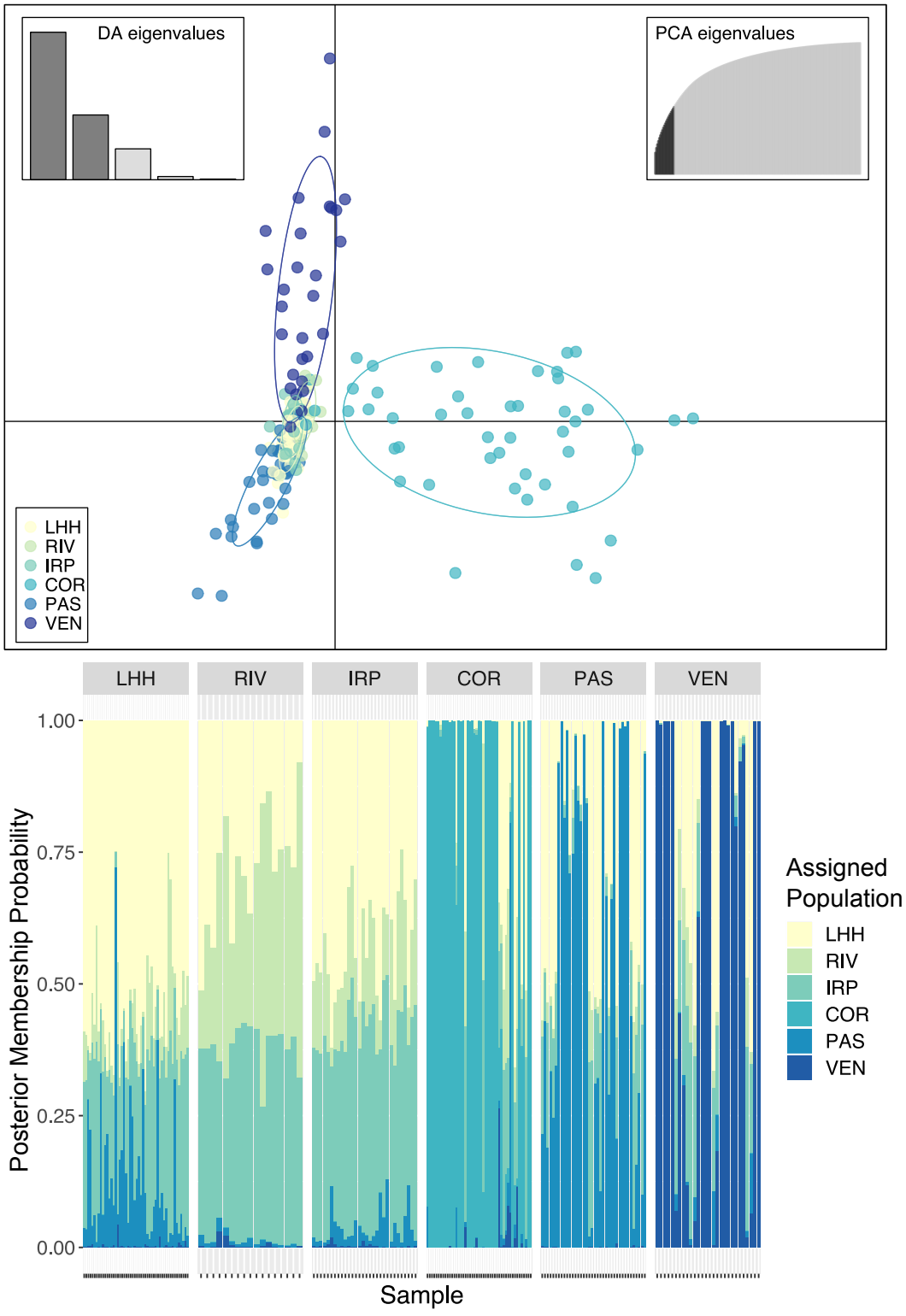
	LHH	**RIV**	**IRP**	**COR**	**PAS**	**VEN**	**ESC**	**SND**
RIV	0.0876							
IRP	0.0692	0.1098						
COR	0.0899	0.0671	0.0821					
PAS	0.0806	0.0901	0.0748	0.0786				
VEN	0.0561	0.1199	0.0687	0.0883	0.0828			
ESC	0.2880	0.2885	0.2879	0.2926	0.3021	0.2764		
SND	0.2737	0.3142	0.2942	0.2777	0.2970	0.2819	0.0578	
IRPK	0.2236	0.3690	0.2807	0.2371	0.2687	0.2742	0.0906	0.0926

PAS, and VEN had high probabilities of correct assignment (Figs. 4.5, S4.8), as did KSHB individuals from all three locations (Figs. 4.6, S4.9) in both relaxed and strict analyses. Each geographic population was also analyzed individually for PSHB, to determine if the different PSHB haplotypes were interbreeding. Posterior probabilities were generally high for individuals of both haplotypes, and revealed little to no admixture between PSHB haplotypes within a locality (Fig. 4.7), indicating that outbreeding between haplotypes is rare.

The inferred number of populations, K, was also estimated from DAPC based on geographical location, and was found to be six for the combined dataset (PSHB + KSHB); six for the PSHB dataset; and three for the KSHB dataset (Fig. S4.4-6). Under the relaxed analysis, K was inferred to be seven for the combined dataset; six for the PSHB dataset; and two for the KSHB dataset (Figs. S4.4-6).

Fig. 4.4 (continued on next page) DAPC (top) and posterior probability assignment of individuals to each population (bottom) of the combined dataset (relaxed analysis). Population of origin is designated at the top, and probability of assignment into each population is designated by color (see legend). Individuals (Sample, x-axis) are denoted by a single vertical bar.


DA eigenvalues
PCA eigenvalues
LHH
RIV
IRP
COR
PAS
VEN
ESC
SND
IRPK
Posterior Membership Probability
1.00
0.75
0.50
0.25
0.00
Sample
Assigned Population


DA eigenvalues
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LHH
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COR
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VEN
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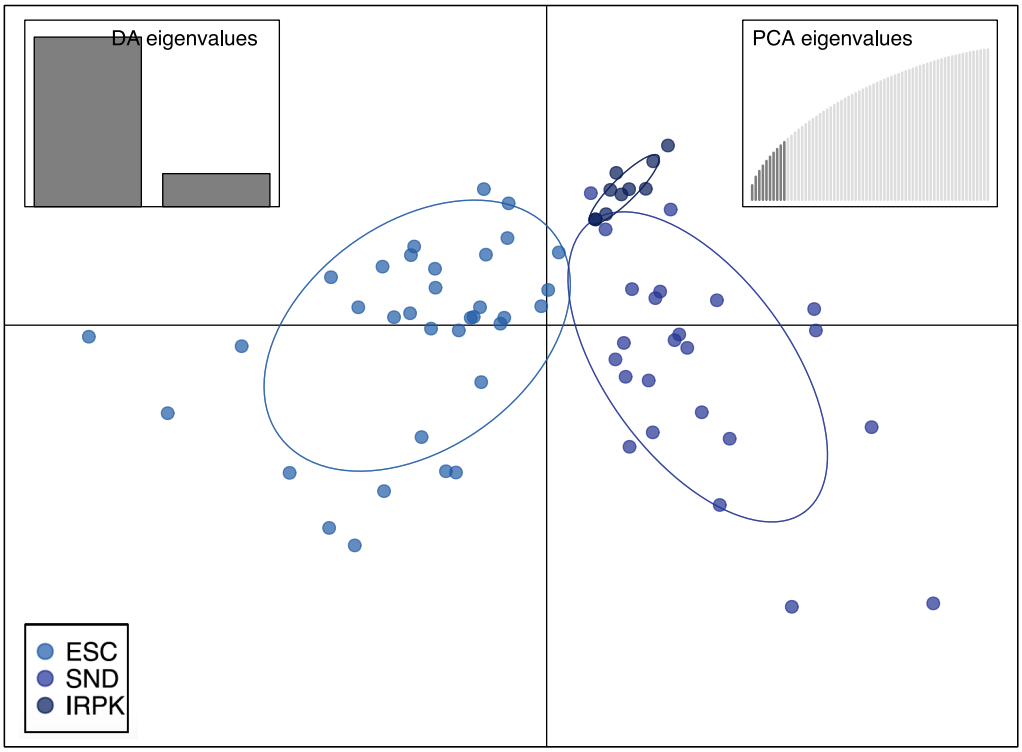
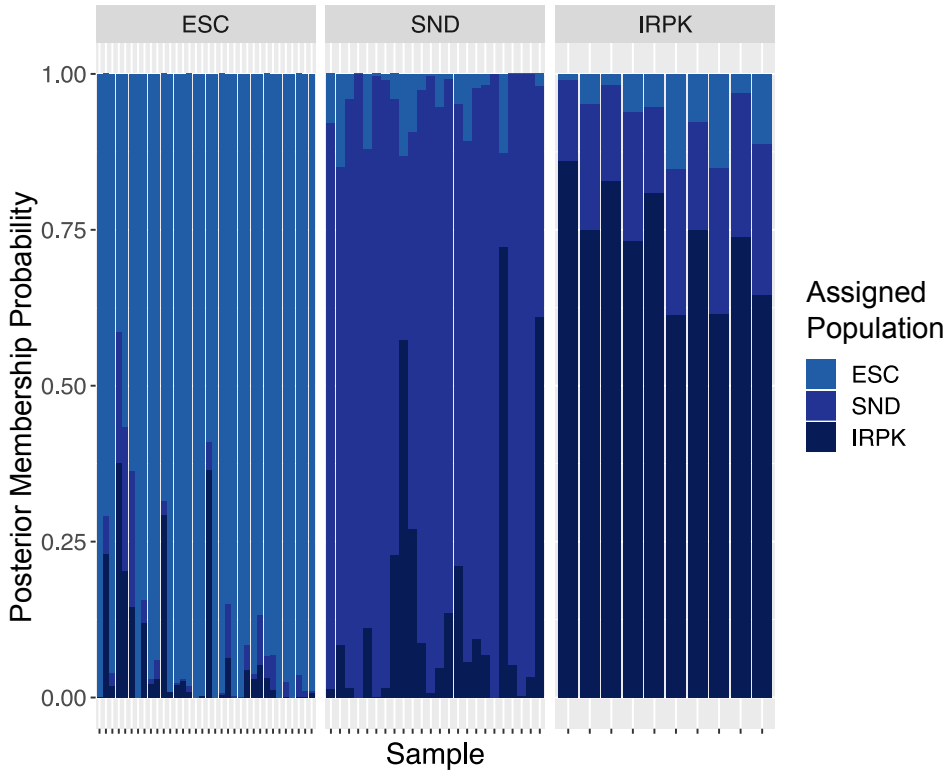
RIV
IRP
COR
PAS
VEN
1.00
0.75
0.50
0.25
0.00
Posterior Membership Probability
Sample
Assigned Population
LHH
RIV
IRP
COR
PAS
VEN

Fig 4.5 (above) DAPC (top) and posterior probability assignment of individuals to each population (bottom) of the PSHB dataset (relaxed analysis). Population of origin is designated at the top, and probability of assignment into each population is designated by color (see legend). Individuals (Sample, x-axis) are denoted by a single vertical bar.

Fig. 4.6 (continued on next page) DAPC (top) and posterior probability assignment of individuals to each population (bottom) of the KSHB dataset (relaxed analysis). Population of origin is designated at the top, and probability of assignment into each population is designated by color (see legend). Individuals (Sample, x-axis) are denoted by a single vertical bar.


DA eigenvalues
PCA eigenvalues
ESC
SND
IRPK


ESC
SND
IRPK
1.00
0.75
0.50
0.25
0.00
Posterior Membership Probability
Sample
Assigned Population
ESC
SND
IRPK

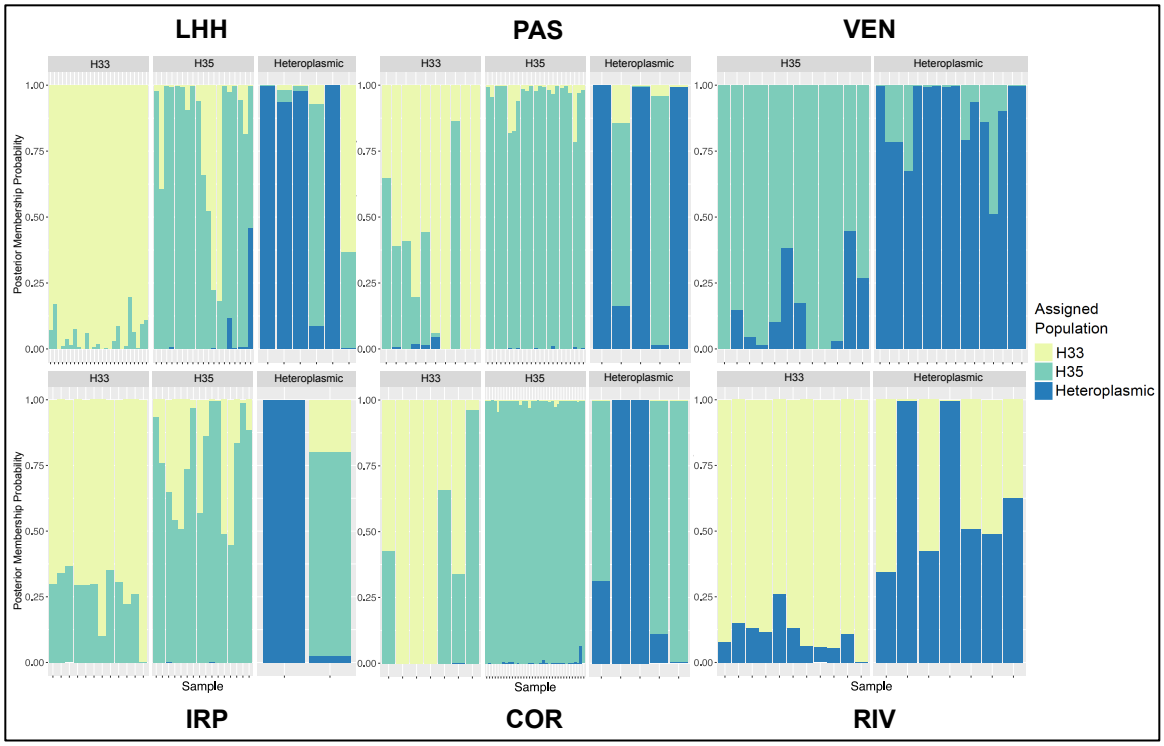


Fig. 4.7 Posterior probability assignment of PSHB to each population based on DAPC, where population is designated by haplotype (relaxed analysis). Analyses were performed separately for each geographic locality. Only H35 and heteroplasmic individuals are present in VEN; only H33 and heteroplasmic individuals were sampled from RIV. Population of origin is designated at the top, and assignment probabilities into different populations are based on color (see legend). Individuals (Sample, x-axis) are denoted by a single vertical bar.

DISCUSSION

We used ddRADSeq to perform the first population genetic analysis on members of the invasive *Euwallacea fornicatus* species complex. Levels of sibmating within PSHB and KSHB populations were lower than anticipated (Table 4.3, Figs. 4.2-4.3), with on average 26% of PSHB females and 39% of KSHB females mating with non-brothers (from strict analysis). This level of outbreeding may be sufficient to prevent population divergence. Inbreeding and sibmating coefficients were lower across loci retained from strict analyses, which required at least half of the individuals in a population to harbor a given locus for it to be retained, compared to relaxed analyses, which required only 15% of individuals in a population to possess the locus (Figs. 4.2-4.3). The number of recovered loci and SNPs per population was also dramatically reduced between relaxed and strict analyses (Table 4.2, Fig. S4.1), but the vast majority of sites from relaxed analyses were homozygous (Figs. S4.2-3), indicating that many of these sites were generated through inbreeding.

We found that KSHB populations are more genetically diverse than PSHB populations in California. This is evidenced by higher heterozygosities and an overall greater number of loci, SNPs, and private alleles catalogued from KSHB populations (Tables 4.2, S4.2; Figs. 4.3, S4.3). Of the PSHB localities sampled, the COR population harbored the most genetic diversity (Table S4.2, Fig. S4.1). We were able to discern geographical population structure using DAPC, which revealed distinct differences between COR, PAS, and VEN populations of PSHB from the relaxed analysis; the remaining PSHB populations were all highly similar to one another, and had low

posterior probabilities of being correctly assigned back into their original location (Fig. 4.5). DAPC of KSHB populations revealed that the IRPK population likely arose recently from individuals originating from the SND population (Fig. 4.6). When considering the entire combined dataset, PSHB and KSHB cluster separately but several KSHB individuals clustered closer to PSHB populations than to KSHB populations (Fig. 4.4). These individuals were genotyped and confirmed as KSHB (data not shown), and may constitute evidence of hybridization between the two species. DAPC using strict analyses resulted in almost perfect posterior probability assignment of individuals back into their original populations (Figs. S4.7-9).

It is important to note that the specimens sampled here represent populations only at a particular moment in time. KSHB is a relatively recent invader in California; to better understand the invasion dynamics of this species, samples collected in future years should be subjected to similar analyses and re-analyzed at a later time. The results presented here should also be compared to those from beetles collected from their native range, where populations have not been heavily influenced by founder effects. The Xyleborini are highly efficient invaders, given that females disperse having already mated inside their natal gallery, and carrying their ambrosia fungi with them inside of specialized mycangia. Even if a female does not mate prior to dispersal, she can produce an all-male brood from unfertilized eggs and then mate with one of her sons to produce another brood of diploid daughters. Xyleborines are thus highly efficient invaders of naïve areas, and it is possible that the invasive SHB populations in California were founded with just one or two foundresses; in fact, it is probable given that only one COI

haplotype of KSHB, and two of PSHB, are known in California, despite the high haplotype diversity present in their native range (Stouthamer et al. 2017). Given that KSHB was discovered in California about a decade later than PSHB, we might expect that current populations of KSHB are more genetically similar than those of PSHB, which may have diversified and diverged as they spread across their invasive range. However, our results suggest that KSHB populations have diversified even more than PSHB, which may constitute evidence of higher rates of outbreeding in this species.

Population density, which may be higher in invasive ranges than native ranges, likely also plays an important role in the interpretation of our results. Higher population densities provide more opportunities for outbreeding, especially since male SHB are known to exit their natal gallery and attempt to enter the galleries of unrelated females (pers. obs.). Although males lack functional wings, they can access other galleries on the same host tree (Fig. 4.8). However, even if males are able to find other galleries, they may be denied entrance by the foundress, as she typically blocks the hole with her abdomen to protect her offspring from predators, parasites, and other external threats (Parthiban 1992). It is unknown how or if foundress entrance-guarding behavior changes as a function of SHB population density in natural settings.

To conclude, although sibmating is the default mating strategy in California populations of PSHB and KSHB, rates of outbreeding were higher than expected, especially in KSHB. Most geographic populations harbor distinct genetic signatures that allow them to be discerned from other populations, and this is especially apparent in results from strict analyses (i.e., those that looked at deeper genetic variation).

Fig 4.8 Five male PSHB surround the entrance to the gallery of an unrelated female, who plugs the hole with her abdomen. PSHB pictured are from colony, reared on bolts of avocado wood. Photo: C. Dodge.

APPENDIX

Table S4.1 PSHB haplotype incidences by locality. Numbers in parentheses represent percentages of total (N).

Location	N	H33 (%)	H35 (%)	Het (%)
Total	**210**	**65 (31.0)**	**105 (50.0)**	**40 (19.0)**
La Habra Heights (LHH)	50	25 (50.0)	19 (38.0)	6 (12.0)
Riverside (RIV)	17	11 (64.7)	0 (0.0)	6 (35.3)
Irvine Regional Park (IRP)	30	12 (40.0)	16 (53.3)	2 (6.7)
Corona (COR)	47	7 (14.9)	35 (74.5)	5 (10.6)
Pasadena (PAS)	38	10 (26.3)	23 (60.5)	5 (13.2)
Ventura (VEN)	28	0 (0.0)	12 (42.9)	16 (57.1)

Table S4.2 Number of private alleles per population.

Population	Strict Analysis	Relaxed Analysis
LHH	0	16
RIV	1	15
IRP	3	18
COR	5	235
PAS	1	70
VEN	1	57
ESC	29	184
SND	14	150
IRPK	3	40

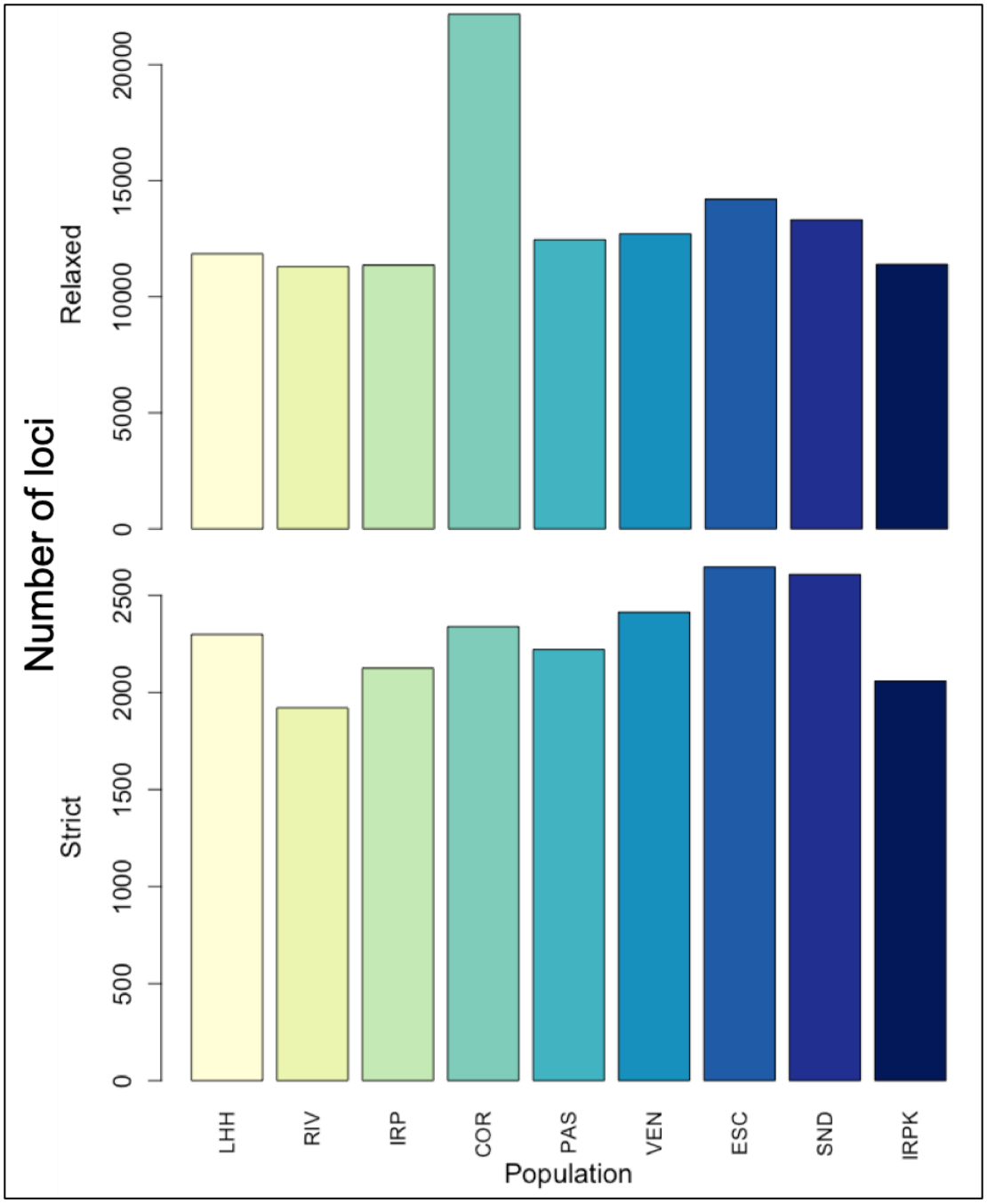


Fig. S4.1 Number of SNPs recovered from each population from relaxed (top) and strict (bottom) *denovo_map* analyses, performed on the entire combined dataset.

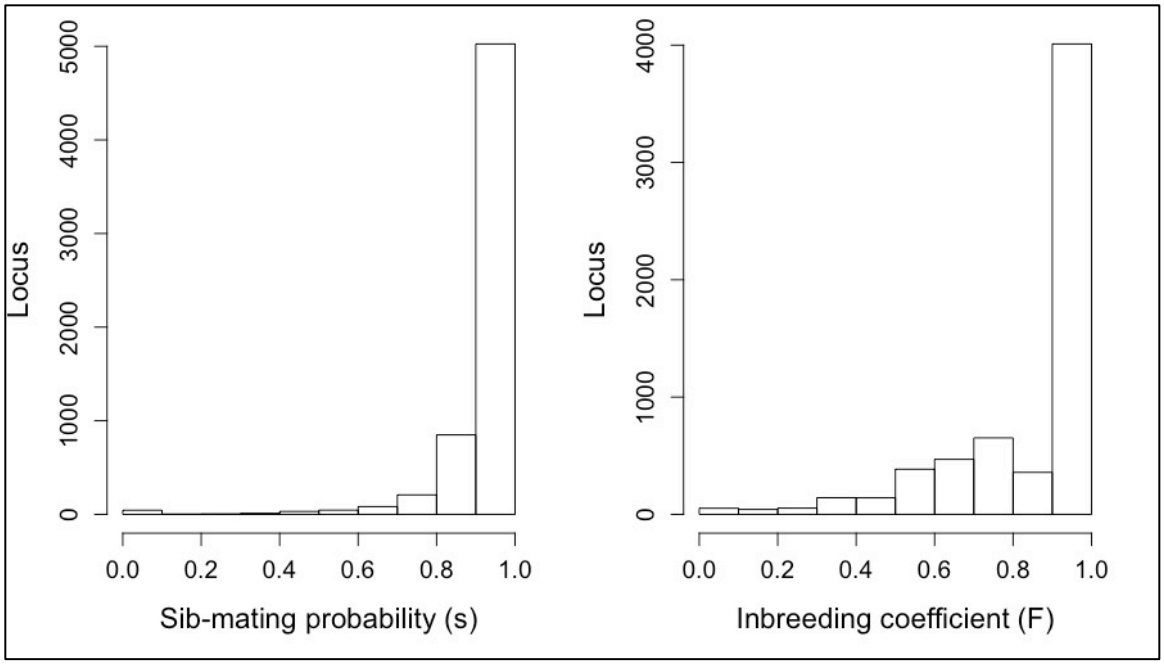


Fig. S4.2 Histogram of sib-mating probabilities (s) and inbreeding coefficients (F) for all recovered loci from PSHB-only analysis (relaxed). A value of 1 indicates that a locus is completely homozygous, whereas a value of 0 indicates that the locus is in HWE.

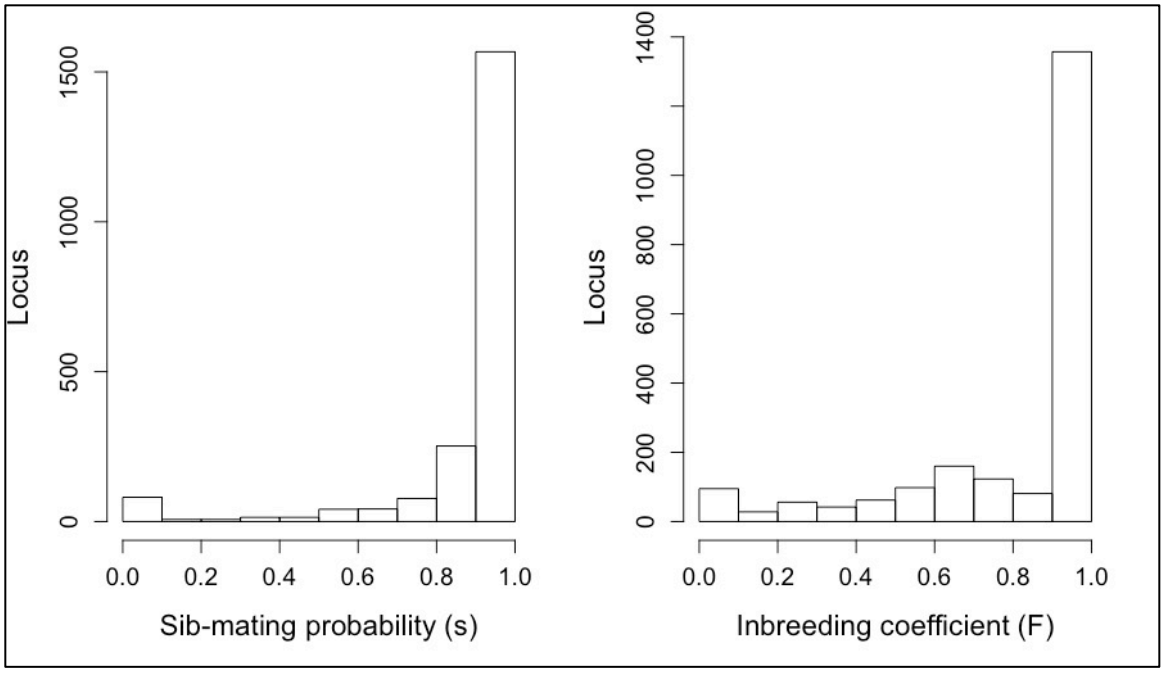


Fig. S4.3 Histogram of sib-mating probabilities (s) and inbreeding coefficients (F) for all recovered loci from KSHB-only analysis (relaxed). A value of 1 indicates that a locus is completely homozygous, whereas a value of 0 indicates that the locus is in HWE.

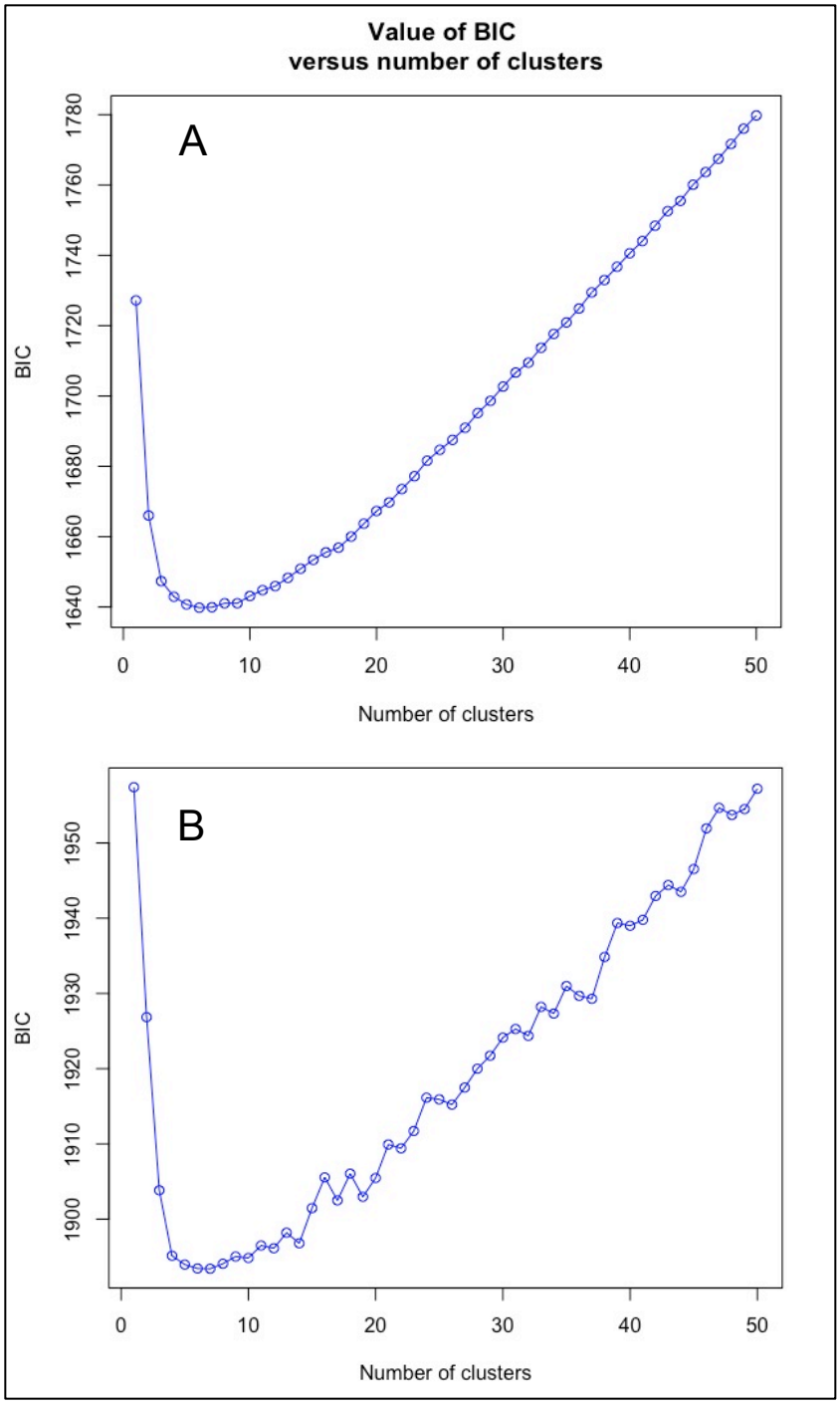


Fig. S4.4 Number of predicted clusters (i.e., populations, K) in the combined dataset, based on k-means clustering, using A) strict and B) relaxed analyses. The most likely value of K is the one with the lowest BIC value.

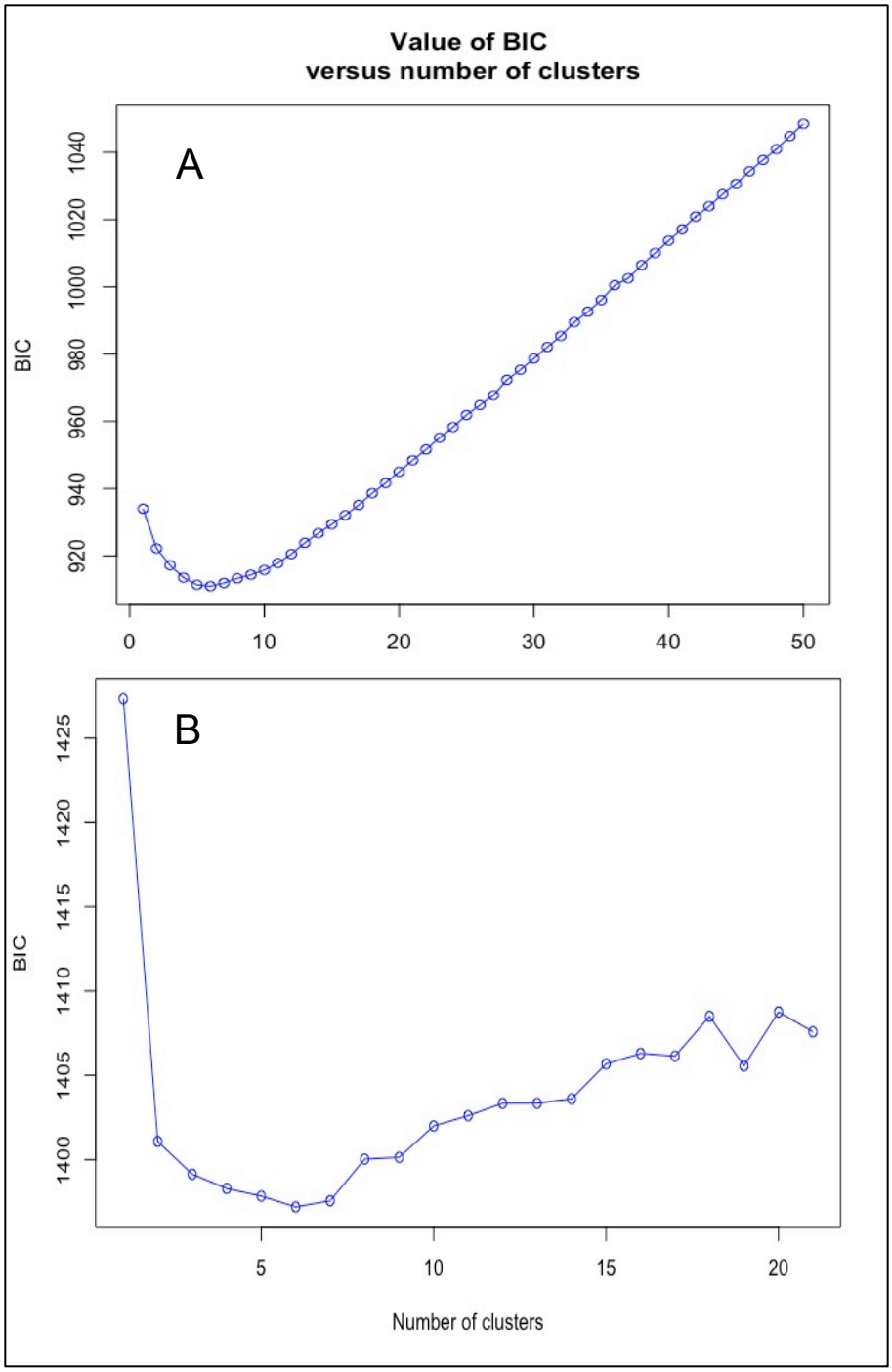


Fig. S4.5 Number of predicted clusters (i.e., populations, K) in the PSHB dataset, based on k-means clustering, using A) strict and B) relaxed analyses. The most likely value of K is the one with the lowest BIC value.

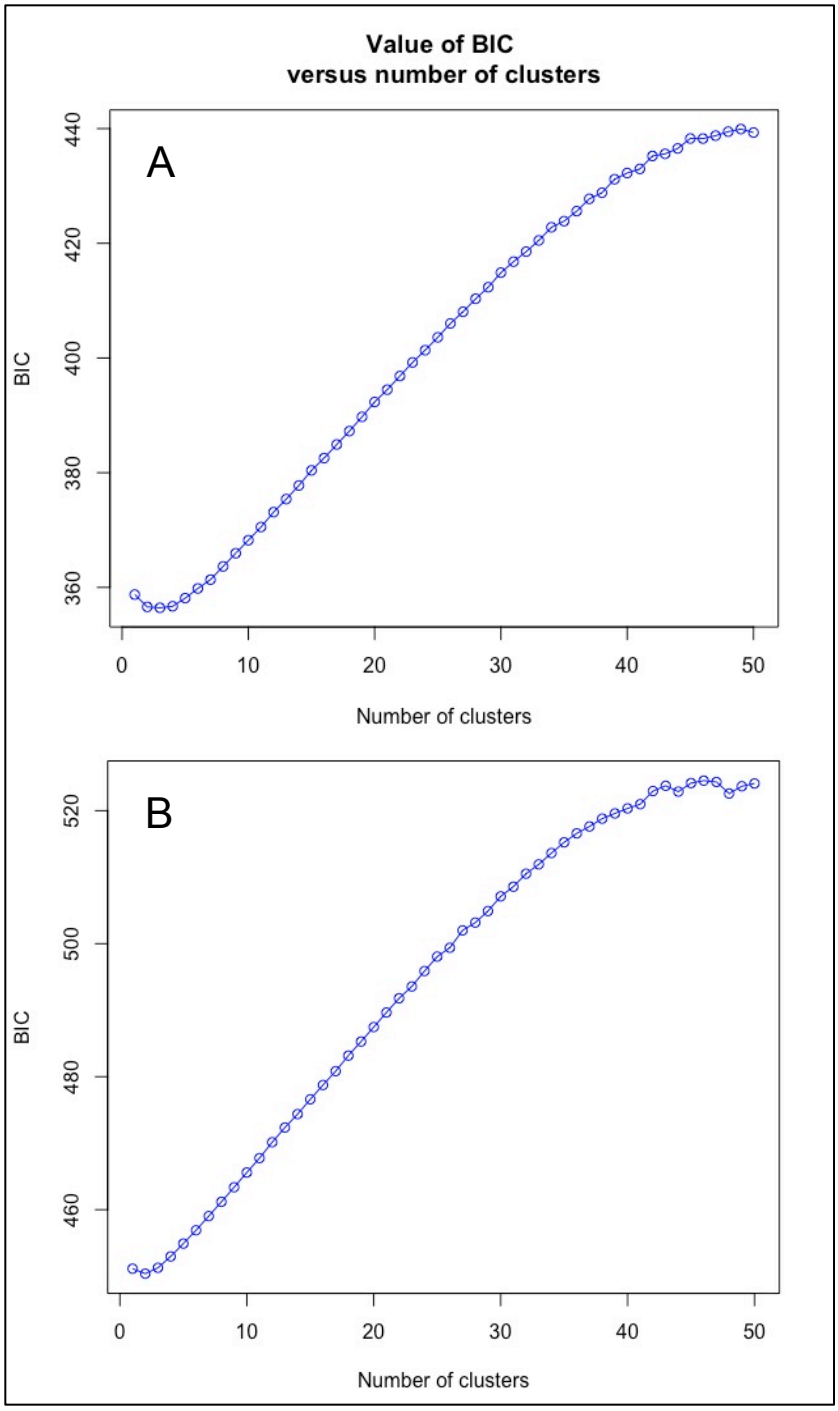


Fig. S4.6 Number of predicted clusters (i.e., populations, K) in the KSHB dataset, based on k-means clustering, using A) strict and B) relaxed analyses. The most likely value of K is the one with the lowest BIC value.

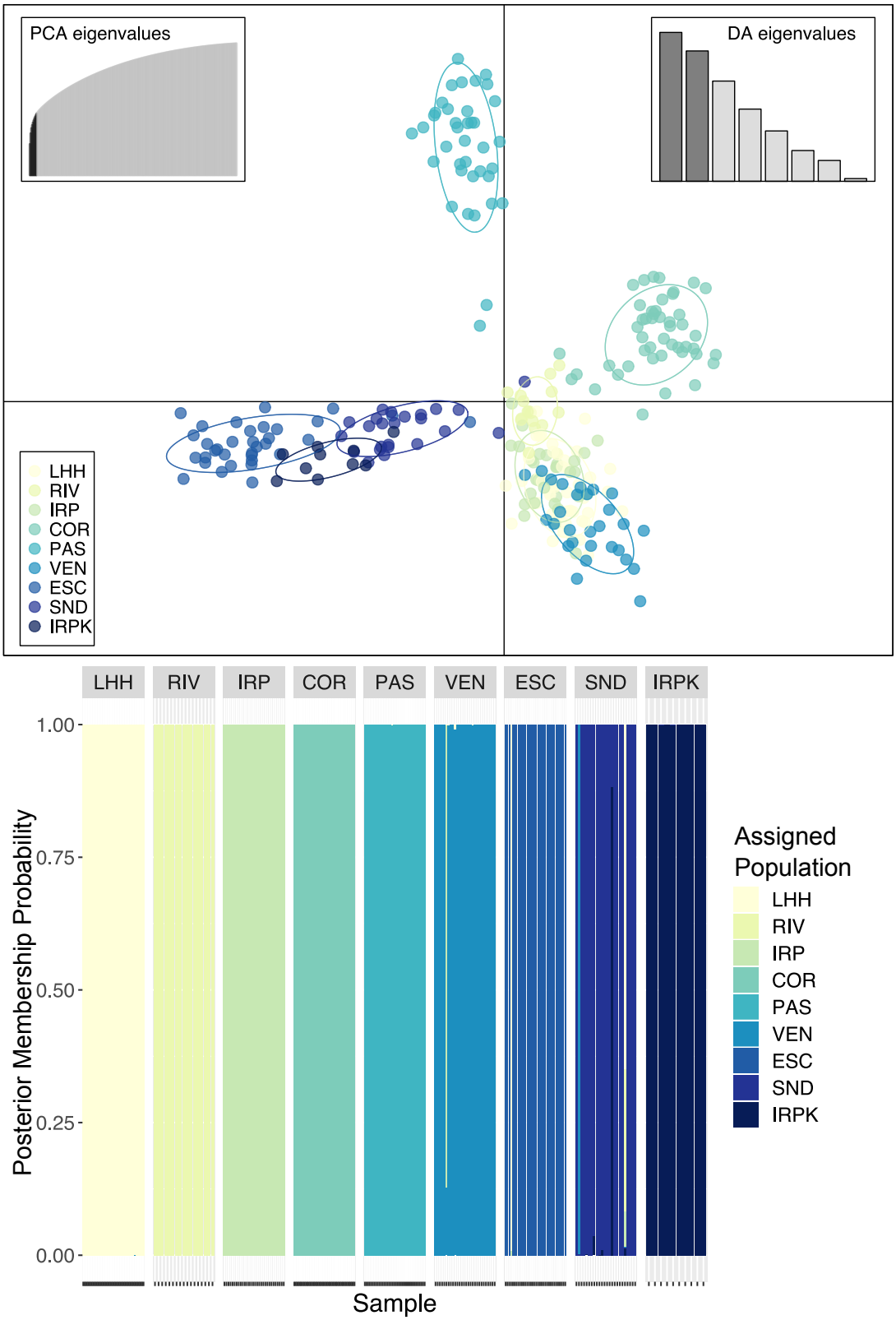
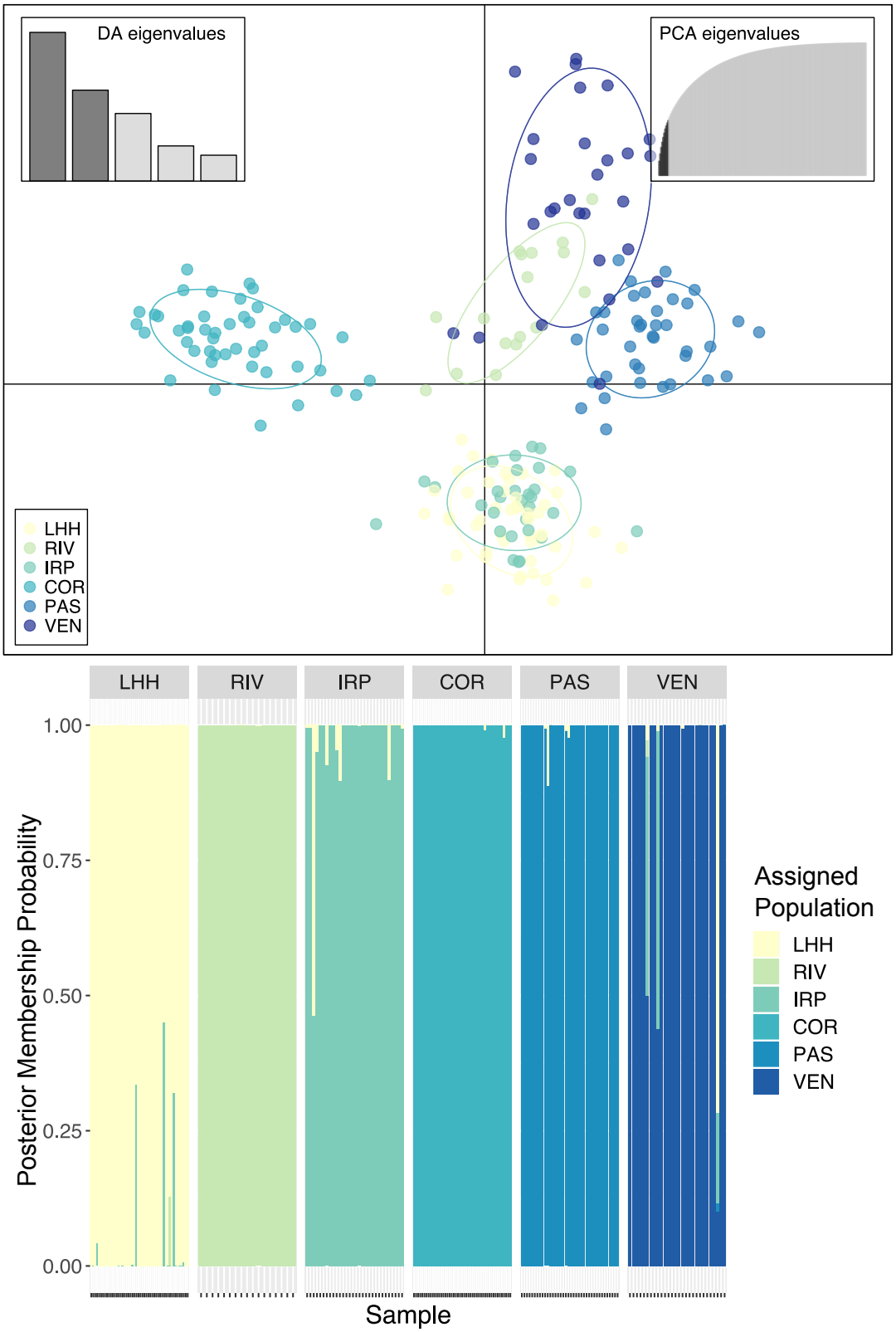

PCA eigenvalues
DA eigenvalues
LHH
RIV
IRP
COR
PAS
VEN
ESC
SND
IRPK
Posterior Membership Probability
1.00
0.75
0.50
0.25
0.00
Sample
Assigned Population

Fig. S4.7 DAPC (top) and posterior probability assignment of individuals to each population (bottom) of the combined dataset (strict analysis). Population of origin is designated at the top, and probability of assignment into each population is designated by color (see legend). Individuals (Sample, x-axis) are denoted by a single vertical bar.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PCA eigenvalues
LHH
RIV
IRP
COR
PAS
VEN
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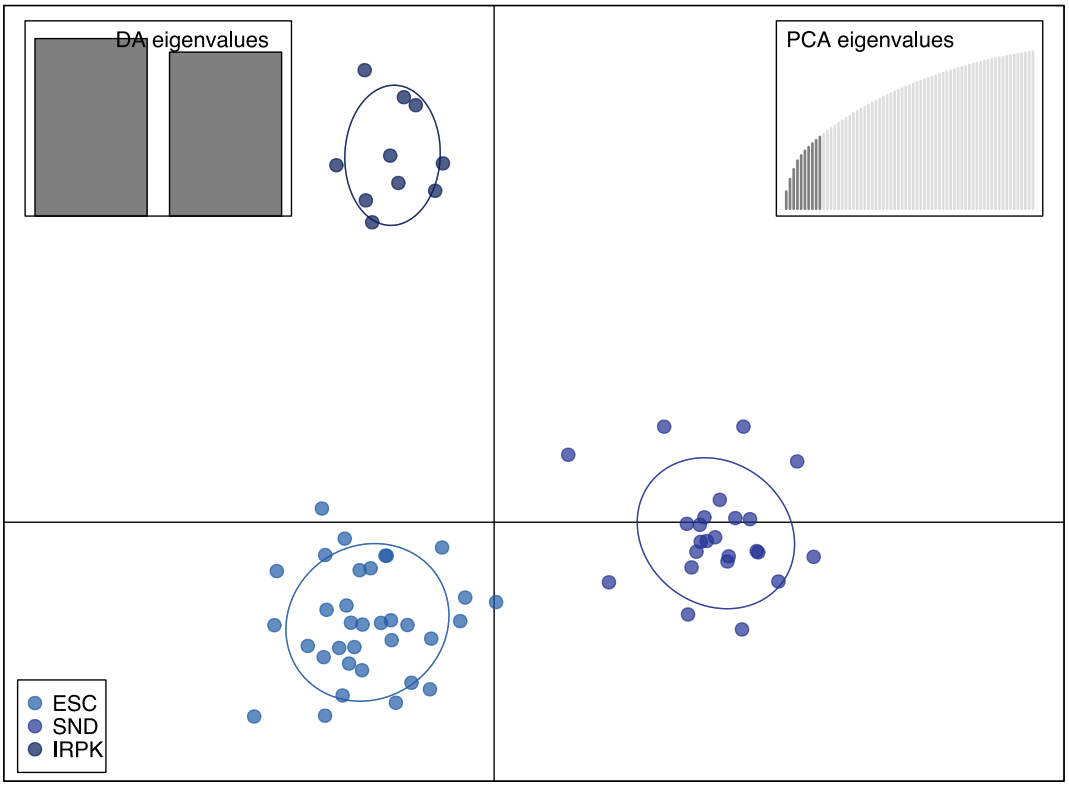
RIV
IRP
COR
PAS
VEN
1.00
0.75
0.50
0.25
0.00
Posterior Membership Probability
Sample
Assigned Population
LHH
RIV
IRP
COR
PAS
VEN

Fig. S4.8 DAPC (top) and posterior probability assignment of individuals to each population (bottom) of the PSHB dataset (strict analysis). Population of origin is designated at the top, and probability of assignment into each population is designated by color (see legend). Individuals (Sample, x-axis) are denoted by a single vertical bar.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PCA eigenvalues
ESC
SND
IRPK

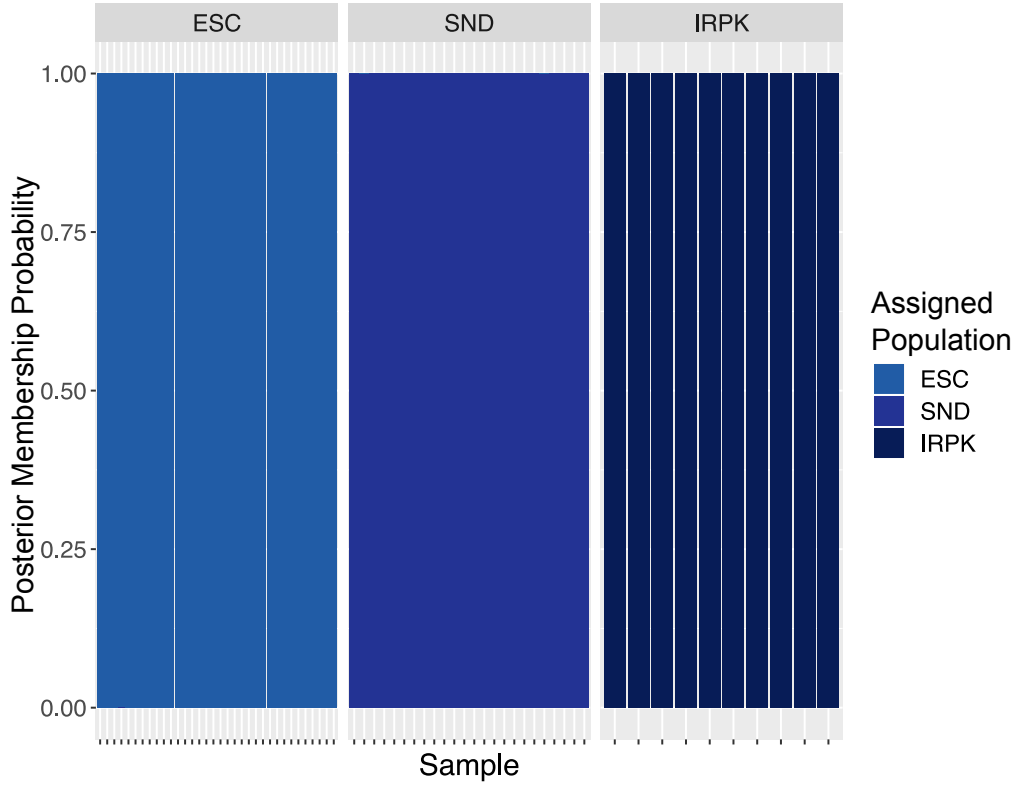
ESC
SND
IRPK
1.00
0.75
0.50
0.25
0.00
Posterior Membership Probability
Sample
Assigned Population
ESC
SND
IRPK

Fig. S4.9 DAPC (top) and posterior probability assignment of individuals to each population (bottom) of the KSHB dataset (strict analysis). Population of origin is designated at the top, and probability of assignment into each population is designated by color (see legend). Individuals (Sample, x-axis) are denoted by a single vertical bar.

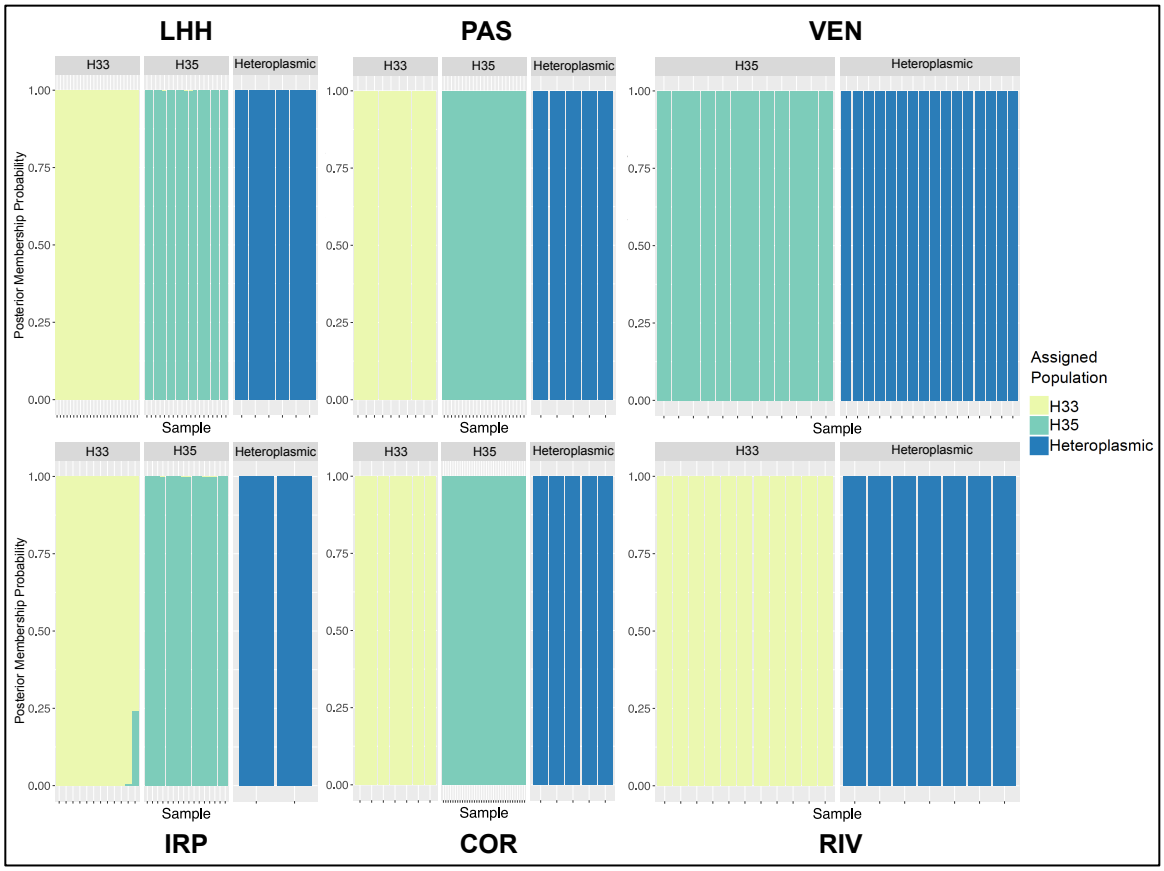

Fig. S4.10 Posterior probability assignment of PSHB, where population is designated by haplotype (strict analysis). Analyses were performed separately for each geographic locality. Only H35 and heteroplasmic individuals are present in VEN; only H33 and heteroplasmic individuals were sampled from RIV. Population of origin is designated at the top, and assignment probabilities into different populations are based on color (see legend). Individuals (Sample, x-axis) are denoted by a single vertical bar.

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