UNIVERSITY OF CALIFORNIA
RIVERSIDE

Host-Plant Mediated Phenotypic Plasticity of Invasive Shothole Borers in Southern California, and Identification of Other Arthropods Associated With Their Habitat

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

Master of Science

in

Entomology

by

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June 2023

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ABSTRACT OF THE THESIS

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by

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University of California, Riverside, June 2023
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The ambrosia beetle species Euwallacea fornicatus Eichhoff and Euwallacea kuroshio Gomez & Hulcr (Coleoptera: Curculionidae: Scolytinae), commonly known as the polyphagous shothole borer (PSHB) and Kuroshio shothole borer (KSHB), respectively, are major pests of ornamental and agriculturally important trees. We were interested to find any morphological differences within the members of the Euwallacea fornicatus species complex, due to them being cryptic species of each other. Since their detection in California in 2003 (PSHB) and 2013 (KSHB), population numbers have been steadily increasing, raising concerns among growers of certain commodities (e.g. avocado) and

those tasked with conserving natural areas. However, in the past few years, the numbers of PSHB and KSHB in once-heavily infested areas appear to have fallen quite dramatically. The reasons for the sudden decrease are unclear, but one possible contributing factor is that a resident natural enemy has adapted to these shothole borers and is consequently affecting a level of control. Another explanation would be boom-bust dynamics, which are common for new invasions of pest populations. Thus, we were interested in documenting and identifying any parasitoids or predators that could potentially be causing a decrease in PSHB and KSHB populations. We collected infested logs from different locations in Southern California, maintained those logs in a controlled environment for several months, and collected everything that emerged from the logs. Emerging insects were collected daily into >95% ethanol and subsequently identified using DNA barcoding. We found mainly Dipterans that feed on fungus or decaying plant materials and a few parasitoids that target the fungus feeders.

Acknowledgements

I would like to first thank my advisor Dr. Richard Stouthamer. I am extremely grateful for his guidance and patience before I even started grade school. I appreciate the energy and hours he has put in to assist me on my research topics. I appreciate Dr. Stouthamer is always pushing me to be a better scientist. Second, I would like to thank Dr. John Heraty for encouraging me to work on different systematic projects. I would like to thank Dr. Timothy Paine for funding my first year of Graduate school. I would like to thank the fellow list of people that are significant to me: Dr. Deena Heusin, Dr. Valeh Ebrahimi, Dr. Christine Dodge, Fang Ling (Chloe) Liu, Amanda Lee, Zackary Balderrama, Lisa Balderrama, Elias Balderrama, Lily Gillespie, and my cohort for being by my side whenever I needed extra encouragement. Last but not least, I am grateful for having my daughter Ino Wu. Her smiles and laughs are my biggest encouragement.

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1. Introduction

The fungus-farming ambrosia beetle *Euwallacea fornicatus* species complex have been important pests of economically important crops and ornamental trees in different parts of the world (Stouthamer et al. 2017). The identification of the members in the species complex is almost impossible relying only on morphological characters. Chapter one will be focusing on the testing of those characters on all members of the *E. fornicatus* species complex with different environmental factors.

Cryptic species has always been a controversial subject within taxonomy. They resemble similar morphological feature of each other and are often harder to identified without using genetic tools. The morphological identification within the ambrosia beetle *Ewallacea fornicatus* (Eichoff) (Coleoptera: Curculionidae) species complex has been in dispute since 1922 (Eggers, 1922; Winn Sampson, 1923). Members of *E. fornicatus* species complex are cryptic species of each other, therefore it is hard to distinguish the morphological features. *Euwallacea fornicatus* was first described as *Xyleborous fornicatus* by Enchoff (1868) and the type specimens were collected in Sri Lanka. Eggers (1922) designated the larger type specimens to *X. fornicatus* and smaller type specimens as *X. fornicatior*. However, since the size of the specimens are not a good morphological character to separate one species from another, different articles argued the correct designation of the beetle (Winn Sampson 1923, Beeson 1930). The different host plants also added to the difficulty of identifying the members within the species complex (Gadd 1942, Judenko 1961). Kalshoven (1958) attempted to send specimens to different taxonomy experts in an attempt to identify the specimens based on morphology, but the

response were in disagreement. More species that were morphologically similar to *X. fornicatus* and *X. fornicatior* including *X. whitfordiodendrus* (Schedl, 1942) *X. perbrevis and X. schulzei* (Schedl, 1951a), and *X. tapatapaoensis* (Schedl, 1951b) were described by Schedl. It was not until Wood (1989) and Wood and Bright (1922) to revise genus Euwallacea that synonymized many of the morphological similar species (*X. whitfordiodendrus*, *X. schulzei, X. perbrevis*, *X. fornicatior)* with *E. fornicatus*. The body size and host preference are still characters that are argumentative with the placement of the beetle species.

With the aid of new technology, we were able to identify specimens with their genetic information. COI sequences were used to identify the beetles that were found as pest attacking trees in California (Eskalen et al 2013, Stouthamer et al. 2017). The beetle pest was identified as *E. fornicatus* morphologically (Eskalen et al 2013). However, since this is a major pest observed in avocado orchards in California, we wanted to learn more on where the origin of those beetles came from. The origin of the pest could help us identify possible natural enemies in the native area that could possibly help with the control of the pest. Stouthamer et al (2017) recognized three or four clades that were considered as the *E. fornicatus* species complex. The three clades were Polyphagous Shot Hole Borer (PSHB), Kuroshio Shot Hole Borer (KSHB), Tea Shot Hole Borer (TSHB) with possible two clades (TSHBa and TSHBb). The native area with most of members of the *E. fornicatus* complex occur is Taiwan. The first chapter were focused on distinguishing if there are any significant morphological characters that are distinguishable within the species complex. We were also interested to see if there are

any environmental factors such as host difference and host size difference that can affect the morphological characters of the species complex.

Since the invasion of SHB in California (PSHB in 2003 and KSHB in 2013), there has be steady increase of SHB population size as expected for new foreign pests. However, recently there have been noticeable drops of PSHB and KSHB populations in California. In chapter two, we examined all of the insects and arachnids that emerged from logs that have evidence of active SHB colonies. We were interested to see if there are any natural enemies that could be affecting California's SHB populations.

2. Chapter 1

Host-plant mediated phenotypic plasticity of invasive shothole borers in Southern California

2.1 Abstract

The *Euwallacea fornicatus* Eichoff species complex consists currently of four recognized species of ambrosia beetle. The identification of the species within this complex has been in flux since 1922. Members of the species complex are thought to be cryptic species with no apparent morphological differences. Thus far, the most accurate and successful way to identify the different species of *E. fornicatus* species complex is using the sequence of the mitochondrial COI gene (Stouthamer et al. 2017). Correlating the DNA data with measurement of morphological characters allowed Gomez et al. (2018) and Smith et al. (2019) to assign the correct taxon to approximately 80% of specimens, using the size of two morphological characters. In this chapter, we show that these morphological measurements do not reliably assign species identity to specimens collected in Taiwan, which falls within the native range of three of the four recognized taxa. *E. perbrevis* individuals were correctly identified in 10 of 22 specimens, *E. kuroshio* was never correctly identified for 22 specimens, while *E. fornicatus* was correctly identified in 24 of 31 specimens. The morphological data would indicate that *E. kuroshio* is not present in Taiwan, while the fourth species *E. fornicatior,*-which has never been found in Taiwan, would be present. The morphological characters used appear to be influenced by the host plant from which the beetles emerge. However, the diameter of the branches (diameter 3-5 cm, 5-10 cm and larger than 10cm) in which beetles develop does

not appear to influence the size of these characters when tested for one species emerging from avocado branches. It is clear that the only accurate method of distinguishing these taxa is the use of the mitochondrial COI sequences.

2.2 Introduction

Identification of the taxa within the species complex of the ambrosia beetle *Euwallacea fornicatus* (Eichhoff, 1868) has been in dispute since 1922 (Eggers, 1922; Winn Sampson, 1923). *Euwallacea fornicatus* was first described as *Xyleborus fornicatus* by Eichhoff (1868) with the type specimens collected from Sri Lanka. Eggers (1922) designated the name *Xyleborus fornicatus* to individuals that were larger, and *X. fornicatior* to smaller individuals, *X. fornicatior* also had a more humped appearance than *X. fornicator*. Several other authorities on ambrosia beetles at that time could not apply these morphological differences in a consistent manner (Winn Sampson 1923, Beeson 1930). The difference between these species remained a topic of discussion, particularly in Sri Lanka where beetles of this species complex were a pest in tea, but also occurred in a number of different host plants (Gadd 1942, Judenko 1961). Kalshoven (1958), collected beetles of this complex in Indonesia and sent out specimens from his collection to several taxonomic authorities to ask them to identify these specimens and found that he could not get a consistent answer placing a specimen in either species. After 1922 several other species were described by Schedl (1942,1951a,b, 1959)) that were very close in appearance to *E. fornicatus* including: *X. whitfordiodendrus* (Schedl, 1942) *X. perbrevis and X. schulzei* (Schedl, 1951a), and *X. tapatapaoensis* (Schedl, 1951b). How these

species could be distinguished from each other was not addressed by Schedl, however Wood (1989) and Wood & Bright (1992a) first revived the genus *Euwallacea* and next placed *Xyleborus fornicatus* in it, and also synonomized many of the closely related species (*X. perbrevis*, *X. fornicatior*, *X. whitfordiodendrus*, *X. schulzei*) with *E. fornicatus*.

When beetles, morphologically identified as *E. fornicatus* were discovered as attacking avocado trees in California (Eskalen et al. 2013), COI sequences were used to determine the origin of the invasion (Stouthamer et al. 2017). Determining where the beetles came from is important to find natural enemies to try to biologically control these invaders. Species identification is extremely important when it comes to biological control. Misidentification of the species targeted for biological control can lead to importing and studying natural enemies that are not specialized on of the target species (see for instance DeBach 1960). After sequencing the mitochondrial COI of the invasive beetles in California and many beetles collected from South East Asia, Stouthamer et al (2017) distinguished three (possibly four) different clades in what they considered the *E. fornicatus* species complex. These clades were identified using the common names polyphagous shothole borer (PSHB), Kuroshio shothole borer (KSHB) and tea shothole borer (TSHB), TSHB harbored two different clades (A and B). Stouthamer et al (2017) hypothesized that the native range of PSHB consisted of Thailand, Vietnam, China, Taiwan and Okinawa, this taxon was invasive in Israel (Mendel et al. 2012), USA (California)(Rabaglia et al. 2006, Eskalen et al. 2013) and most recently in South Africa (Paap et al 2018) and Australia (Cook & Broughton, 2023). TSHB is native to Southeast

Asia, and has invaded Florida, and Hawaii (Carrillio et al. 2012, Rugman-Jones et al. 2020). KSHB's native range consists of Taiwan, Okinawa, and Indonesia and is invasive in USA (California) (Eskalen et al. 2013, Stouthamer et al. 2017). Gomez et al. (2018) used a set of specimens identified using COI sequences that were obtained from Stouthamer et al (2017) supplemented with additional specimens from the Hulcr lab at the University of Florida. For these specimens a large number of morphological traits were measured. Following a Classification and Regression Trees (CART) statistical analysis on this set of characters from 89 specimens, two characters were sufficient to correctly identify 80% when applied to the specimens used in their data set. The two characters that allowed the identification were the length of the pronotum and the length of the elytra. Morphological characters were also measured for the type specimens of the species that had been synonymized by Wood (1989). This included all the species with the exception of *E. fornicatus*, type specimens for this species were thought to have been lost in World War II (Wood &Bright, 1992). The measurements of the type specimens were then used to assign the type specimens to one of the DNA based clades. Following this exercise the PSHB clade was associated with the name *E. whitfordiodendrus*, the TSHBa clade with *E. fornicatus*, TSHBb with *E. fornicatior* and finally the KSHB clade was described as a new species with the name *E. kuroshio* (Gomez et al. 2018). Shortly following this publication, a type specimen for the species *E. fornicatus* was discovered and measured leading to a revision of the names (Smith et al. 2019) associated with the PSHB DNA cluster, it changed from *E. whitfordiodendrus* to *E. fornicatus*, while the TSHBa clade changed from *E. fornicatus* to *E. perbrevis*. The identification method

using the morphology remained the same for the DNA based clusters only the scientific name associated with the cluster changed.

Here we test the morphometric method of identification of specimens of the *E. fornicatus* species complex on beetles collected in Taiwan. In Taiwan three of the four recognized species co-occur. In addition, we determine several environmental factors that may influence the size and shape of the beetles. These factors include plant host from which the beetles emerge and the diameter of the branches in which the beetles mature.

2.3 Materials and methods

2.3.1 General collection of host plants and SHB

Three species of shot hole borer and a basal haplotype in the PSHB clade identified as haplotype 22 are present in Taiwan (Stouthamer et al. 2017). Specimens were collected from wood of different host plant species in Taiwan from September to December 2020 (Table 1). Hosts plants sampled were *Litchi chinensis* (Lichi), *Castanea mollissima* (Chinese Chestnut), *Persea americana* (Avocado), *Camellia sinensis* (Tea), and *Ricinus communis* (Castor Bean). Wood materials was collected in Taiwan with proper permits and shipped by express mail to the University of California, Riverside Insectary Quarantine facility. Upon arrival in the facility logs were placed in individual glass or plastic containers with mesh tops in rooms with 12 hrs of light and 12 hrs of dark, temperature at 30°C to 31.67°C, and the humidity was maintained at 45 to 50 percent. Emerging beetles were collected daily and placed into 95% ethanol.

2.3.2 Comparing different SHB species

Initially the accuracy of the method develop by Gomez et al 2018 and Smith et al 2019, using morphometric data to identify the species of the *E. fornicatus* species complex, was tested by comparing the identity of the beetles using the morphometric data with the clade identity provided by the COI sequence of the specimens. Beetle collection was done in two ways, either by allowing beetles to emerge from plant host material, or by collecting beetles from sticky traps (Scentry Wing Traps, 23×28 cm, Jen Yung Industrial, Ltd., Taiwan) placed in different orchards in Taiwan (Liu et al. 2022).

Branches collected in the field were brought back to the lab, where the cut ends of the branches were covered with parafin wax to slow down the drying out of the branches. Branches were place in bugdorms (MegaView Science Co., Ltd., Taichung,Taiwan) in a rearing room with 12hrs:12hrs light and dark, 26°C (± 2°C) at 70% humidity. Each day emerged individuals were removed and placed in 95% ethanol.

The individuals caught on the sticky traps originated from a Lichi orchard in Fenyuan Township, Changhua County in November 2020. Each sticky trap was equipped with one Quercivoral lure (ChemTica Internacional, Heredia, Costa Rica). Traps were placed in the field for two weeks, and collected on 24th November 2020. Each card was examined and the number of shot hole borers was recorded. Specimens were removed from the sticky traps using a single droplet of HistoClear II (National Diagnostics, Atlanta, GA, USA). After removal of the specimens from the trap card, they were placed into HistoClear again for one minute to dissolve any remaining adhesive residue. Specimens were subsequently washed with sterile water and placed in 95% ethanol.

The DNA of both the lab-emerged and trapped specimens was extracted using the hotSHOT method (Truett et al. 2000; Rugman-Jones et al. 2020). We identified the trap-collected specimens using a high resolution melt (HRM) assay. This method distinguishes between the different taxa of this species complex with a taxon specific melting curve (see Liu et al. 2022, Rugman-Jones and Stouthamer 2017, Liao et al. 2023). This method consists of amplifying a 132 bp fragment of the mitochondrial COI that differs between the taxa recognized as TSHB, KSHB, PSHB and H22. After amplification of the DNA the resulting product was slowly heated up (from 68℃ to 85℃

in 0.1 ℃ increments) to determine the melt curve of the PCR product in the presence of the fluorescent dye EvaGreen.

The amplification and analysis of the PCR product were done on a Qiagen's Rotor-Gene Q 2plex HRM qPCR machine under the following conditions. qPCR mix per reaction includes 0.4μM of each primer [TW-SHB-HRM-for (5'-CGAACCGAATTAGGAACACC-3') and TW-SHB-HRM-rev (5'-CCAGTTTCCAAATCCACCAA-3')], 4 μM 5x HRM mastermix, and 13.2μM of ddH2O. After the qPCR was aliquoted to the individual tubes , 2μM of DNA extraction was added, resulting in a total of 20μM per reaction. The DNA was amplified using the StepOnePlus™ System (Applied Biosystems, Foster City,California) with the steps: 15 min at 95℃, followed by 35 cycles of 95℃ for 20 sec, 53℃ for 20 sec, and 68℃ for 30 sec. Fluorescent signals were recorded at the end of the step (68°C for 30 sec). High resolution melt (HRM) was performed after the amplification was complete, in which reactions were heated from 68℃ to 85℃ in 0.1 ℃ increments. At the end of the species-specific melt-curves (Figure 1) allowed the identification of all the collected SHB specimens by comparing its melt-curve with the positive controls (previously identified by sequencing (see Liu et al. 2022).

The morphological characters were measured using a Leica image system with a Z16 APO A microscope and 1.0x eyepiece. A drop of clear lubricant (KY Jelly, Reckitt, Parsippany, NJ, USA) was used to immobilize the specimens with the head facing to the left in the glass well (2.0 mm diameter) filled with 95% ethanol during the imaging process. Specimens were placed in lateral and dorsal positions (Figure 2). Images

produced using this method were subsequently used to determine the length of different body parts. The following sizes were determined: body length (dorsal), pronotum length (dorsal and lateral), and elytra length (dorsal and lateral) because these measurements were determined to be the best in separating the different members of the *E. fornicatus* species complex. (Smith et al. 2019: Gomez et al. 2018).

2.3.3 Comparing the impact of different plant host species on SHB size

To exclude influence of beetle taxon on the size of the beetle we chose plant hosts known to harbor beetles with haplotype 22. The different hosts collected were *Castanea mollissima* (Chinese Chestnut), *Camellia sinensis* (Tea), and *Ricinus communis* (Castor Bean). The branches were shipped to UCR quarantine facility and maintained as described above. For each host plant 15 specimens were obtained. The rearing, collection, identification, and measurement process were the same as previously described. Specimens emerging from these branches were identified by HRM analysis, followed in some cases by an additional verification of the HRM results by the sequencing of outlier specimens with the smallest or largest morphometric measurements.

2.3.4 Different branch size experiment (natural setting)

For the different sized branch experiment, we collected *P. americana* (avocado) branches in different sizes at the same location on 20 January 2022 (See Table 1). We focused on collecting three different diameter branches, 3cm- 5cm, 5cm - 10 cm, and >10cm. We were interested to determine if host branch diameter could also affect the beetle size

emerging from these branches. The shipment, rearing, identification, and measurement procedure were the same as the previous experiment. Specimen emerging from these branches were identified using HRM and only PSHB individuals were used in the analysis.

2.3.5 Different rearing tube size experiment (artificial setting)

We were also interested to see if there will be any morphological difference in a more controlled setting, when the diameter of the rearing environment is manipulated. This was done by rearing the beetles on artificial medium in tubes of different diameters (15ml falcon tube inner diameter 1.5cm, 50 ml falcon tube inner diameter 2.77 cm). The 50 ml falcon tubes were filled with 25 ml of diet and the15 ml falcon tubes received 10 ml of diet. To each tube a single mated female was added to produce offspring. The emerged offspring were preserved in 95% ethanol and subsequently measured.

The artificial diet consisted of 65 g avocado sawdust, 20 g of agar, 10 g of sucrose, 5 g of cornstarch, 5 g of casein, 5 g of active dry yeast, 1 g of Wesson's salt mixture. The dry ingredients were mixed thoroughly first before adding the wet ingredients consisting of 2.5ml of wheat germ oil, 5 ml of 95% ethanol, and 500 ml of deionized water. After mixing all the wet and dry ingredients, we placed the media in an autoclave at 121°C for 30 minutes and let it cool down to about 80°C - 65°C for pouring. 50 ml tubes get 25 ml of media and 15 ml tubes get 7.5 ml of media. Twenty SHB were randomly selected from both size tubes. The rearing, identification, and measurement procedure were the same as the previous experiment.

2.4 Data analysis

Data from beetle measurements were analyzed using RStudio (version 4.1.2). We used PCA or Principle component analysis to look at the relationship of the beetles in body length, pronotum length, pronotum width, elytra length, and elytra width. We set up the measurement data as figure (***) in excel and imported in Rstudio. Below is the code for getting to the PCA plot.

Figure 3. How the measurement data converted in from excel to Rstudio. We chose to used PCA plot because it reduces the dimension of the plot to a 2D view, which allows us to easier understand the relationship between the measured specimens without losing too much information.

Codes for the members of E. fornicatus species complex (Figure 6)

```
# Inserting excel data to Rstudio
library(readxl)
NMDS_1 <- read_excel("~/Desktop/SHB project/data sheets/NMDS_1.xlsx")
View(NMDS_1)
# Getting the PCA plot
library(stats)
library(caret)
library(ggfortify)
pca_res <- prcomp(NMDS_1[,-1], scale. = T)
autoplot(pca_res, data = NMDS_1, frame.colour = 'Species', frame=T, label.size
=3)
```

Codes for PCA Biplot of E. fornicatus species complex (Figure 7)

```
#PC1 vs. PC2
ggbiplot(pca_res, choices = c(1,2), obs.scale = 1, var.scale = 1  ,
        groups = NMDS_1$Species, ellipse = TRUE,
        labels = row.names(data),
        varname.size = 3,
```

```
        circle = FALSE) + scale_color_discrete(name = '') + labs(title = "PCA biplot
for SHB species complex") + theme(legend.direction = 'horizontal',
                                  legend.position = 'top')

 #PC1 vs. PC3
 ggbiplot(pca_res, choices = c(1,3), obs.scale = 0.5, var.scale = 1,
         groups = NMDS_1$Species, ellipse = TRUE,
         labels = row.names(data),
         varname.size = 4,
         circle = FALSE) + scale_color_discrete(name = '') + labs(title = "PCA biplot
 for SHB species complex (PC1 vs. PC3)") + theme(legend.direction = 'horizontal',
                                   legend.position = 'top')
```

2.5 Results

2.5.1 Specimen identification: COI sequence vs morphometrics

Results (Fig. 4) show that the measurements of the specimens using the morphometric method differed from the identification obtained by the HRM method. Of the 22 TSHBa (HRM) individuals ten of the specimens would be assigned to TSHBa (Morph), ten to TSHBb (Morph) and 2 to PSHB (Morph). Of the 31 PSHB (HRM) specimens, 24 are assigned to PSHB (Morph), 6 to (TSHBa (Morph) and 1 TSHBb (Morph), of the 22 KSHB (HRM) specimens none were assigned to KSHB (Morph), 11 were assigned to PSHB (Morph) and 6 to TSHBa, and 5 to TSHBb. Finally of the 23 H22(HRM) specimens, 22 were assigned to PSHB (Morph) and 1 to TSHBa (Morph)

2.5.2 Different host experiment

In our previous section, we noticed a trend of measurement variation between the specimens that emerged from different host plants. We were interested to see if different host plants could affect the beetles' sizes. Since PSHB, KSHB, and TSHB could have multiple haplotypes. Beetles with Haplotype H22 were collected from three different hosts that harbored beetles with haplotype 22 (H22) SHB. Table 2 shows the results of the measurement of H22 specimens derived from different hosts.

2.5.3 Different Size branch experiment

PSHB specimens emerging from the smallest branches were slightly larger than the ones that emerged from the bigger branches (>5cm and >10cm in diameter) (Table 3).

2.5.4Different rearing tube size experiment (artificial setting)

Specimens collected from the bigger tubes (50 ml) were slightly, but significantly larger than the ones collected from the 15 ml (Table 4), and all other size measurements also showed significant differences between the different tube diameters, with the exception of elytra width.

2.5.5 PCA to determine differences between the species based on morphological traits

From our Principle component analysis graph (figure 6), we can see that it is not possible to completely separate the members of *E. fornicatus* species complex. From the two PCA biplots (figure 7), we could see that pronotum length, body length, and elytra length were strongly correlated. In the PC1 vs. PC2 graph, all of the variables were almost equally represented, whereas in PC3 vs. PC1, body length/body width was less represented than the other variables. PC1 to PC3 are able to show over 93% of the variation within the different characters.

It is obvious from the PCA analysis (figure 6) that there is considerable overlap between the species when the morphological characters are used to try to optimally separate the different species.

2.6 Discussion

Taiwan is in the native range of at least three of the recognized species *E. prebrevis* (TSHBa), *E. kuroshio* (KSHB) and *E. fornicatus* (PSHB), as well as a fourth as yet unplaced taxon H22 (Stouthamer et al 2017). Gomez et al (2018) and Smith et al (2019) developed a method to identify the different taxa in the *E. fornicatus* species complex using a relatively simple set of measurements: the length of the elytra and the length of the pronotum both measured diagonally. The method based on these measurements could identify the specimens in their data set with an 80% accuracy. Here we tested this morphometric method on a sample of beetles collected from the field in Taiwan. For the situation in Taiwan the morphometric method turned out to be less precise than in the sample used by Gomez et al (2018). Our results show that the morphometric measurement did much worse on the Taiwanese beetles, 24 of 31 PSHB were correctly identified, none of the 22 KSHB was correctly identified and only 10 of 22 TSHBa were correctly identified. For a total of correct identifications of less than 50% (34 out of 73 specimens). Using the morphometric method we would have concluded that TSHBb was present in Taiwan, even though we have never identified a specimen using COI sequences from Taiwan. We would also conclude that KSHB did not occur in Taiwan while it is present.

We also used the morphometric data set to determine if a PCA analysis could give a better separation of the species and the results shows that the analysis does not result in a clear separation of the four taxa. We found the most important characters were pronotum, elytra, and body length from the PCA biplots, which was also found by

Gomez et al. 2018. The best separation resulting from the PCA plot is between TSHB and H22. KSHB and PSHB were not distinguishable in the PCA plot.

To determine which factors might influence the size of the beetles we collected beetles that had grown up in different host plant species. For this we chose host plants that harbored the H22 haplotype. This haplotype is not found in all host plants we have collected from in Taiwan, but it is the only haplotype found in Castor bean, in addition to other species H22 is also found emerging from tea and chinese chestnut. Analysis of the size of the specimens emerging from these three different host plants shows that the host plant influences the size of the beetles. Beetles from Chinese chestnut were bigger than those from castor bean and tea, and those emerging from castor bean and tea did not differ significantly in size. The overall size difference was due to difference in elytra length but not length of the pronotum. Beetles from this species complex use a wide range of host plants and within the host plants different sized branches. The curvature of galleries created inside branches depends to some extent on the diameter of the branch, with the smaller the diameter the more curved the gallery will be. We did not find an influence of the diameter of the branch from which beetles merge on the measures used for morphometrics. In contrast the rearing of beetles on artificial medium in tubes with different diameter did show significant differences in some of the traits used in the morphometric identification. Significant differences were found in overall length, pronotum width and length as well as in elytra length but not the elytra width. Where there were significant difference the means were larger for the vials with the larger diameters. In addition to the diameter differences between the two vial types the vials also contained different amounts of artificial medium. It is difficult to determine if the diameter of the vial or the amount of food available to larvae inside the vial, or some other factor associated with diameter was important in determining the

differences in the morphometric character values. What is clear however from these different experiments that the values of the morphometric characters are influenced by different environmental factors making their application for species identification of field collected specimens unsuitable.

2.7 Figures and Tables

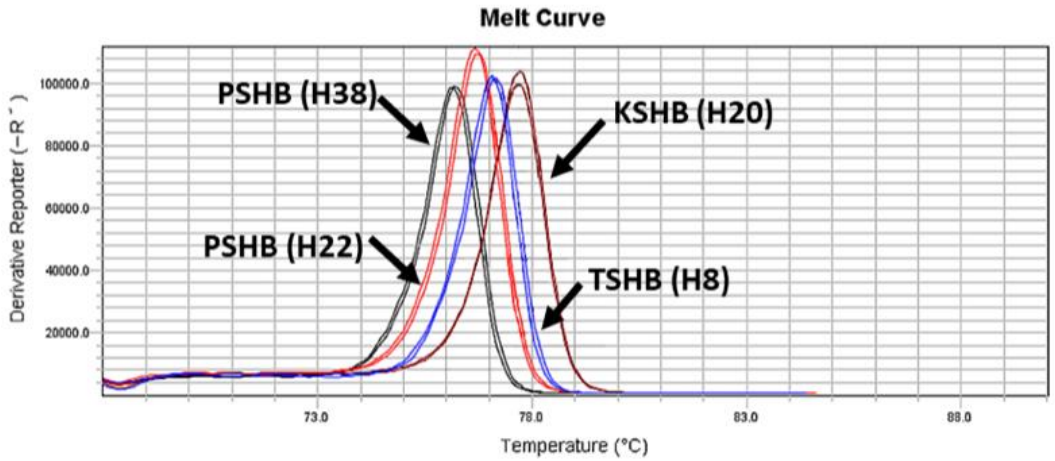

Figure 1. High resolution melt (HRM) curves for identifying different species in SHB species complex. Image taken from Liu et. al 2022.

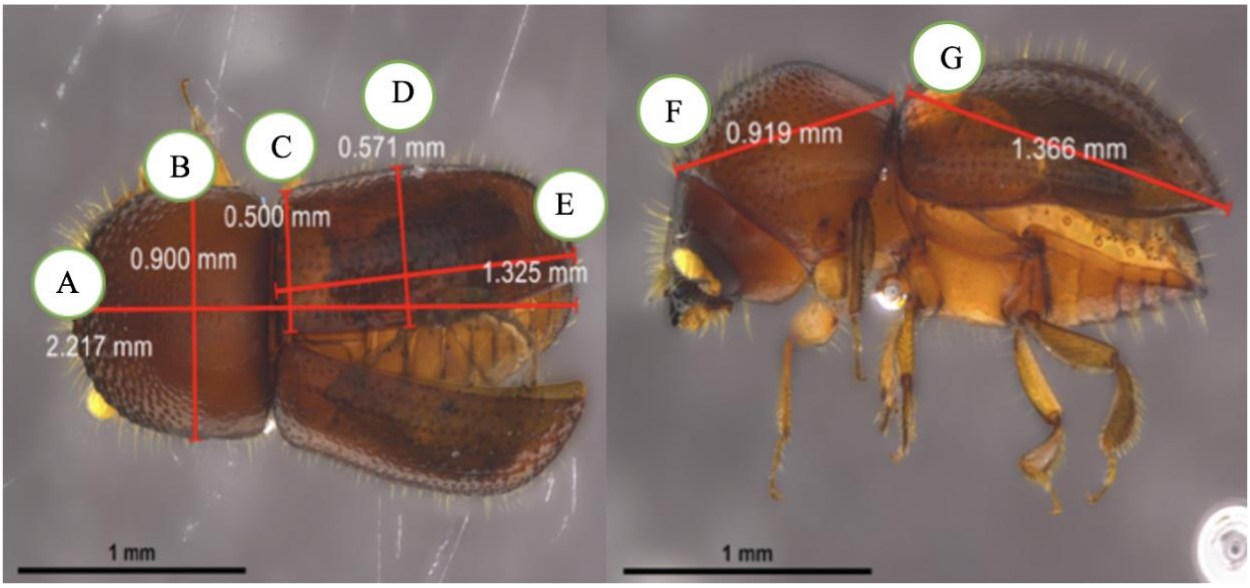

Figure 2. Example of how the specimens were imaged and measured (Specimen PR21121_H22) Left being dorsal view and right being lateral view. A) Body length, B) Pronotum Dorsal, C) Elytra Dorsal Thin, D) Elytra Dorsal Thick, E) Elytra dorsal length, F) Pronotum lateral, G) Elytra lateral.

Filter

	Species	length (mm)	Elytra width (mm)	Elytra length (mm)	pronotum length (mm)	body length/ body width	body width
1	PSHB	2.524	0.568	1.551	1.078	2.221831	1.136
2	PSHB	2.599	0.569	1.607	1.124	2.283831	1.138
3	PSHB	2.503	0.574	1.549	1.091	2.330540	1.074
4	PSHB	2.347	0.477	1.563	1.110	2.218336	1.058
5	PSHB	2.464	0.478	1.564	1.104	2.371511	1.039
6	PSHB	2.357	0.524	1.484	1.078	2.249046	1.048
7	PSHB	2.486	0.522	1.505	1.067	2.381226	1.044

Species	length (mm)	Elytra width (mm)	Elytra length (mm)	pronotum length (mm)	body width
PSHB	2.524	0.568	1.551	1.078	1.136
PSHB	2.599	0.569	1.607	1.124	1.138
PSHB	2.503	0.574	1.549	1.091	1.074
PSHB	2.347	0.477	1.563	1.11	1.058
PSHB	2.464	0.478	1.564	1.104	1.039
PSHB	2.357	0.524	1.484	1.078	1.048
PSHB	2.486	0.522	1.505	1.067	1.044
PSHB	2.476	0.491	1.558	1.104	1.071
PSHB	2.597	0.48	1.599	1.146	0.96
PSHB	2.563	0.515	1.596	1.107	1.03

Figure 3. How the measurement data converted in from excel to Rstudio. We chose to used PCA plot because it reduces the dimension of the plot to a 2D view, which allows us to easier understand the relationship between the measured specimens without losing too much information.

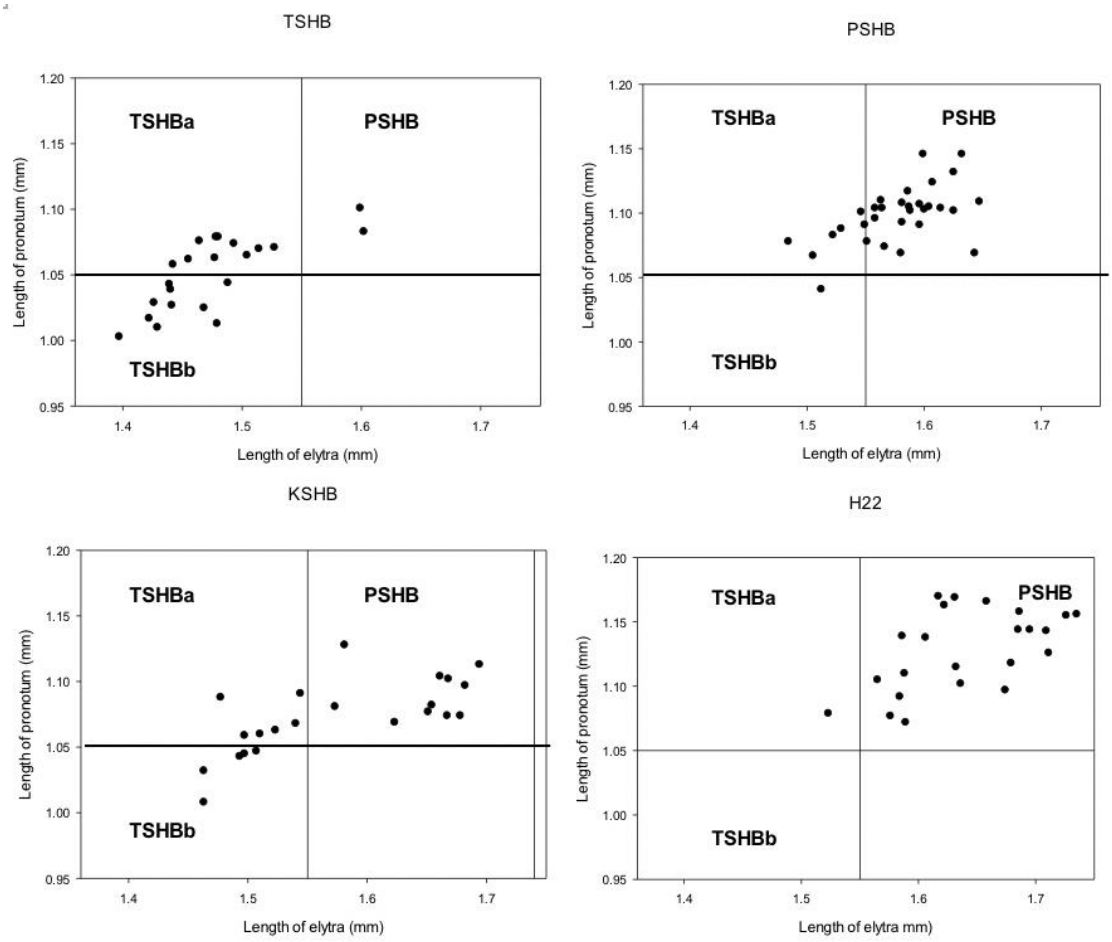

Figure 4. Identification of specimens of *E. fornicatus* species complex using the HRM technique based on difference in COI between the different species, and the morphometric method of Gomez et al (2018) and Smith et al (2019). In this method the length of the elytra and the length of the pronotum should identify the specimens with a accuracy of 80%. The plots for each HRM identified taxon is divided in at least 4 sections by lines indicating the demarcation for the different taxa using the morphometric method. The points indicate the measurements for individuals. In the graph for HRM identified KSHB individuals an extra demarcation line is shown. The morphometric method identifies KSHB individuals when they have a pronotum length larger than 1.05mm and an elytral length larger than 1.74 mm.

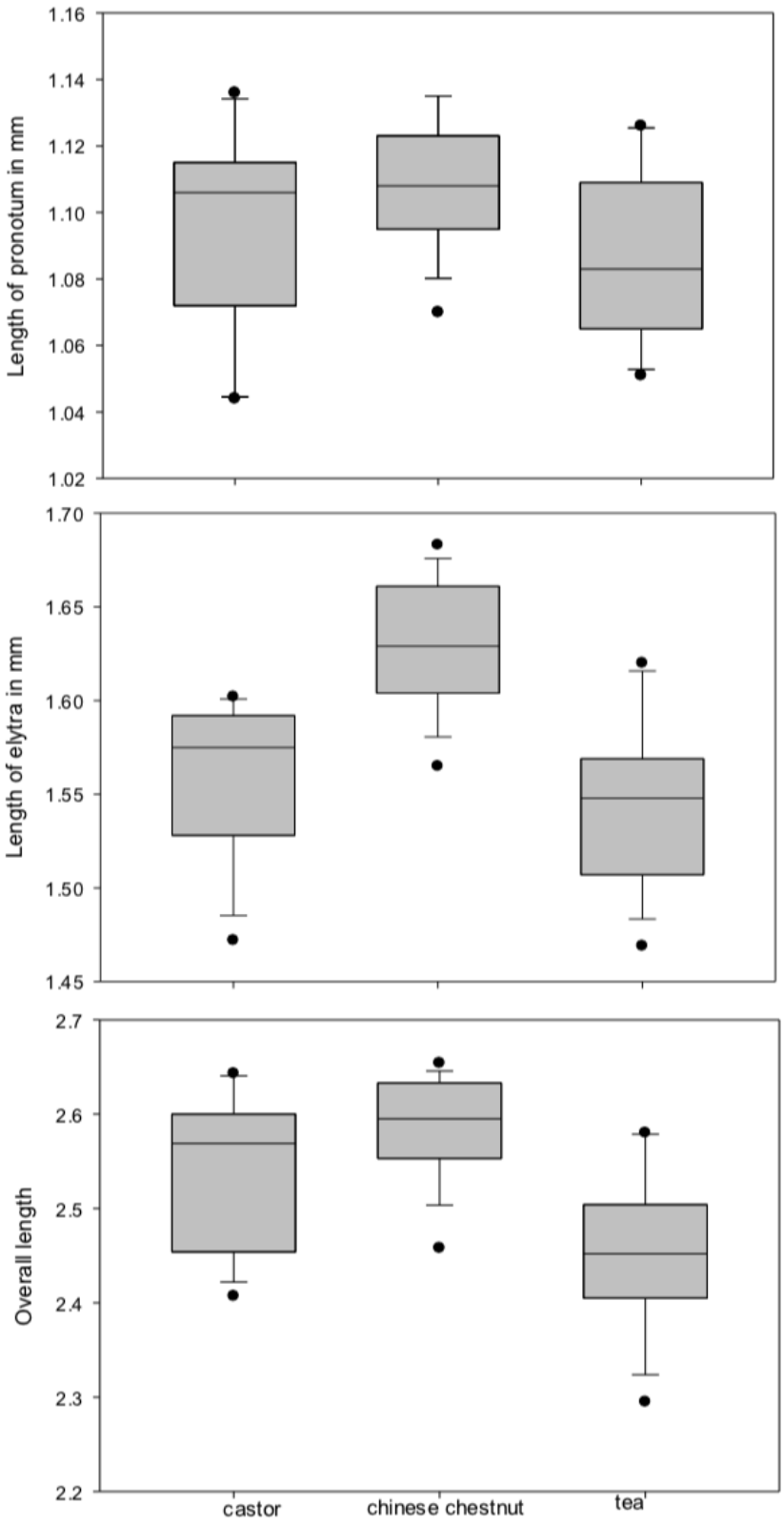
Length of pronotum in mm
1.16
1.14
1.12
1.10
1.08
1.06
1.04
1.02
Length of elytra in mm
1.70
1.65
1.60
1.55
1.50
1.45
Overall length
2.7
2.6
2.5
2.4
2.3
2.2
castor
chinese chestnut
tea

Figure 5. Boxplot for H22 specimens emerged from castor, chinese chestnut or tea wood. Values are shown for the length of the pronotum, the length of the elytra and the overall length of the beetles.

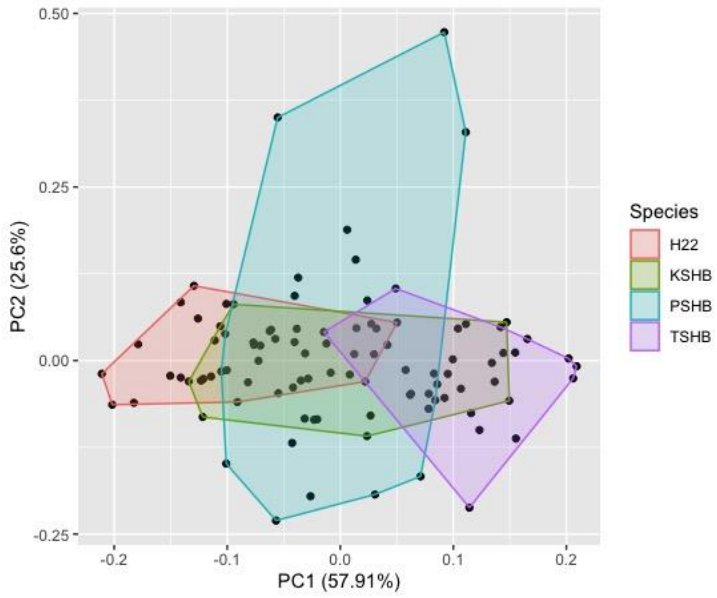

Figure 6: Principle Component Analysis for the *E. fornicatus* species complex. principle component 1 (x-axis) percentage represents the best linear line of the combinations of the variables, which are body length, pronotum length, elytra length, pronotum width, and elytra width is enough to explain 57.91% of the variation that is tested between the four species. PC2 is the second best linear line that consist of all variables and explained 25.6% of the variation. The total variation is 83.51%.

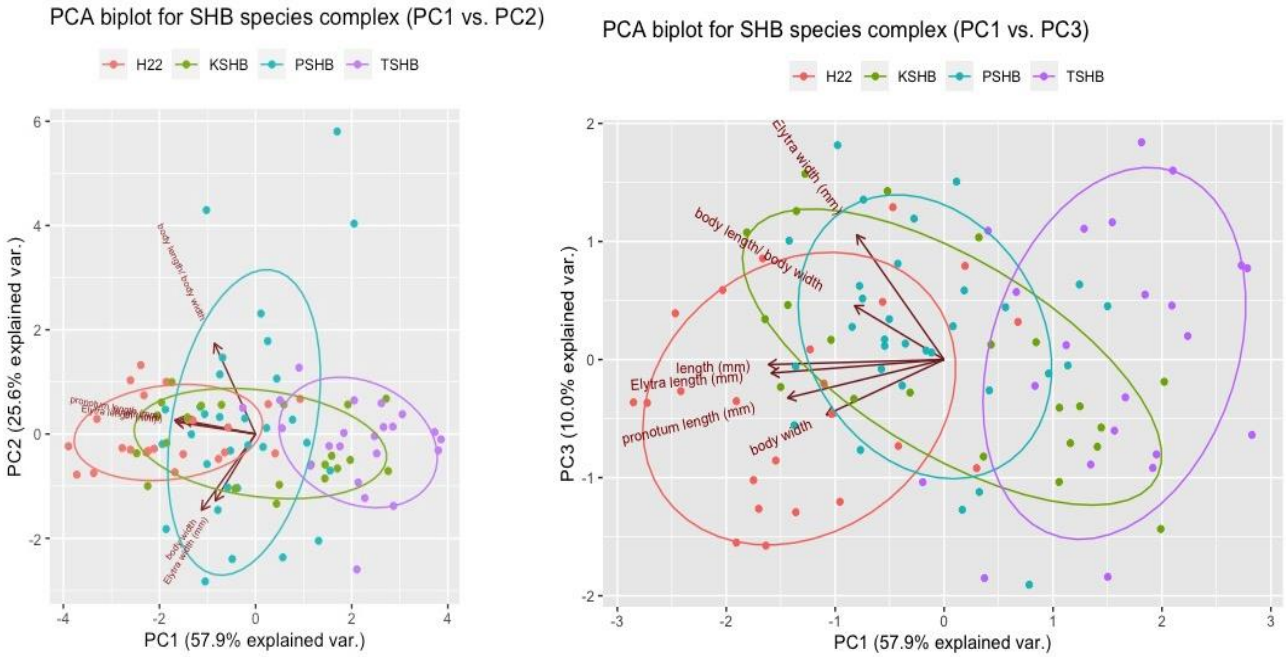

Figure 7. Two PCA biplots of the *E. fornicatus* species complex measured illustrating the effect of the variables on the principle components. A. PCA 1 versus PCA2 and B. PC3 vs. PC1, All the variables contributed about equally to the difference between species in the PC1 vs. PC2 graph.

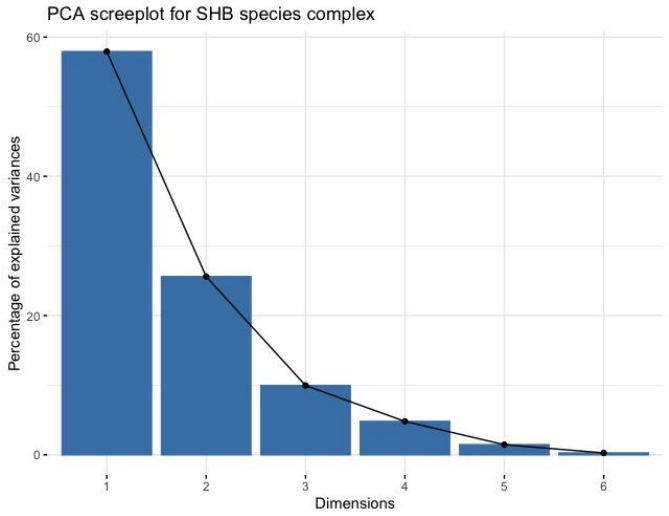

Figure 8: Screeplot of different members in *E. fornicatus* measured. Over 93% of the variations were explained from PC1 to PC3.

Table 1. Taiwan collection data. Plant host species and location of wood collected for beetle emergence. Emerged beetles were identified using the HRM method and morphometric data were collected to use for their morphological identification.

Host collected	City	Location name	Coordinates	date
Camellia sinensis	Nantou County	Zhushan Township	23.728183, 120.691684	24-Nov-20
Camellia sinensis	Hualien County,	Ruisui Township	23.448746, 121.355052	19-Dec-20
Camellia sinensis	Nantou County	Mingjian Township	23.877216, 120.644611	26-Nov-20
Castanea mollissima	Changhua County	Dazhang Rd, Fenyuan Township	23.977999,120.619664	1-Sep-20
Litchi chinensis	Changhua County	Fenyuan Township	24.023306, 120.59919	24-Nov-20
Persea americana	Tainan City	Danei District	23.144393, 120404804	24- Nov-20 20-Jan-22

Table 2. Host plant ID – castor bean vs Chinese chestnut vs tea. Analyzed using oneway-ANOVA and Sidak post-hoc pairwise comparisons (indicated by lowercase letters).

Character	Castor bean	Chinese chestnut	Tea	$F_{2,42}$	p
Pronotum length	1.096 ± 0.008	1.108 ± 0.005	1.087 ± 0.007	2.81	ns [0.0715]
Elytra length	1.560 ± 0.010 **(a)**	1.629 ± 0.009 **(b)**	1.544 ± 0.011 **(a)**	20.18	<0.0001
Overall length	2.538 ± 0.021 **(a)**	2.586 ± 0.013 **(b)**	2.453 ± 0.021 **(a)**	12.74	<0.0001

Table 3. Mean and standard error of morphological characters measured (in mm) of H22 specimens emerging from field collected avocado branches with different diameter:- <2 cm vs 5 cm vs >10 cm, analyzed using oneway-ANOVA.

Character	**<2 cm**	**5 cm**	**>10 cm**	$\mathbf{F_{2,31}}$	**p**
Elytra width	0.493 ± 0.006	0.493 ± 0.003	0.492 ± 0.006	0.02	ns [0.9805]
Elytra length	1.591 ± 0.015	1.585 ± 0.010	1.577 ± 0.014	0.23	ns [0.7986]
Pronotum width	1.060 ± 0.007	1.048 ± 0.005	1.048 ± 0.007	1.24	ns [0.3023]
Pronotum length	1.090 ± 0.008	1.076 ± 0.006	1.075 ± 0.006	1.45	ns [0.2504]

Table 4. Mean and standard error of PSHB beetles emerging from Falcon tubes with different diameter (15 mm versus 27 mm containing artificial diet (10ml versus 25 ml resp.) Means analyzed using a t--tests.

Character	**Mean 15 mm (± s.e.)**	**Mean 50 mL (± s.e.)**	**t**	**df**	**p**
Body length	2.490 ± 0.014	2.526 ± 0.011	-2.0374	37	0.0488
Elytra width	0.502 ± 0.003	0.508 ± 0.003	-1.3689	37	ns [0.1793]
Elytra length	1.553 ± 0.007	1.576 ± 0.006	-2.4029	37	0.0214
Pronotum width	1.071 ± 0.004	1.084 ± 0.004	-2.2403	37	0.0312
Pronotum length	1.093 ± 0.006	1.116 ± 0.005	-3.2093	37	0.0027

3. Chapter 2

Identification of other arthropods associated with their habitat

3.1 Abstract

The ambrosia beetle species *Euwallacea fornicatus* Eichhoff and *Euwallacea kuroshio* Gomez & Hulcr (Coleoptera: Curculionidae: Scolytinae), commonly known as the polyphagous shothole borer (PSHB) and Kuroshio shothole borer (KSHB), respectively, are major pests of ornamental and agriculturally important trees. Since their detection in California in 2003 (PSHB) and 2013 (KSHB), population numbers have been steadily increasing, raising concerns among growers of certain commodities (e.g. avocado) and those tasked with conserving natural areas. However, in the past few years, the numbers of PSHB and KSHB in once-heavily infested areas appear to have fallen quite dramatically. The reasons for the sudden decrease are unclear, but one possible contributing factor is that resident natural enemies have adapted to these shothole borers and are exerting a level of control. Another explanation would be boom-bust dynamics, which are common for new invasions of pest populations. Thus, we were interested in documenting and identifying any parasitoids, predators or competitors that could potentially be causing a decrease in PSHB and KSHB populations. We collected infested logs from different locations in Southern California, maintained those logs in a controlled environment for several months, and collected everything that emerged from the logs. Emerging insects were collected daily into >95% ethanol and subsequently identified using DNA barcoding. No clear single predator or parasitoid was found in the different locations that could explain control of the beetle populations. Consequently, the factors

that may lead to the control of these populations may be a developing “community resistance” or be caused by pathogens that may be affecting the beetles. Community resistance may be caused by the many potential competitors we identified. Unfortunately, we did not study the presence of pathogens or nematodes.

3.2 Introduction

The fungus farming ambrosia beetle *Euwallacea fornicatus* Eichhoff (Coleoptera: Curculionidae: Scolytinae (common name Polyphagous Shot Hole Borer, PSHB) was first detected in California in 2003 (Rabaglia *et al.*, 2006). This invasive species has a wide range of host plants with most of them being ornamental and native hardwood trees (Eskalen et al 2013). One of which is an agriculturally important crop, avocado. Newly emerged mated females will carry fungal spores from their natal galleries and spread them to the newly excavated tunnels. The spores will grow in the new gallery and the beetle larvae and adults use the fungi as their food source. Thus, the cycle will continue. Male shot hole borers usually stay in their natal gallery for their whole life. There is no successful area-wide control method for the beetle population at this moment.

Shot hole borer populations remained largely unnoticed in Southern California until about 2012 when a heavily infested backyard avocado tree was inspected and found to be harboring ambrosia beetles (Eskalen et al 2013). Following the detection and identification of the beetles, surveys were done in Southern California to determine the extent of the invasion. While initially the beetle appeared to be limited to parts Los Angeles County, it soon was shown that the infested area was larger and since then the beetles have spread throughout most of the Southern California Counties. In several areas the infestation reached high numbers both in street trees and in commercial avocado groves. In addition to the species *E. fornicatus* (Smith et al 2019), a second closely related species, *Euwallacea kuroshio* (Common name Kuroshio Shot Hole Borer, KSHB), was detected in 2013 in San Diego county in both avocado groves and in riparian

areas. In one of these riparian areas, the Tijuana River Valley, the beetle population was studied in detail and reached extremely high numbers and in the process killed many willow trees (Boland, 2019). In the year five report of KSHB in Tijuana River Valley, Boland and Uyeda (2020) report the rapid population growth and subsequent decline. KSHB was first observed in the Tijuana River Valley in 2015 and since that time has been estimated to kill more than 120,000 willows in the valley (Boland 2019). After reaching very high numbers the population of beetles collapsed, potentially because the suitable host trees had been killed. Since that time the tree population has recovered, however, there was no major reinfestation (Boland and Uyeda, 2020). The reason for the decrease in population size is currently unknown. A similar pattern was observed along the Santa Clara River by Bennett (2020), where the population of beetles increased rapidly to decline to almost undetectable levels. Not only are such rapid increases and subsequent equally rapid declines reported from the invasion in Southern California, but they have also been observed in the native range of these shot hole borers. Kalshoven (1958a,b) reports several cases where an outbreak of shot-hole borers lasted for 2-3 years and then disappeared.

Rapid increases followed by sharp decreases in population numbers have been observed frequently in invasive species, and have been named boom-bust cycles. The boom-bust dynamics is defined as an increase in population numbers to an outbreak level and followed by a sudden decrease (Strayer et. al. 2017). Boom-bust dynamics could be a single or a recurring event, it is typically found in invasion ecology. The reason for the rapid decline in these cycles could be competition, natural enemies, or limited host

resources, but are often not known. No reason for the rapid decline in the population of shot hole borers in Southern California is known, here we determine which other insect species are found in association with wood infested by shot hole borers and use their known food sources to determine if these species could play a role in the rapid decline of the beetle population.

3.3 Materials and methods

3.3.1 Infested log collection and specimen rearing

To find different insects that are associated with shot hole borer in their habitat, we collected infested branches and logs from various locations in Southern California. The host species that we focused on were *Salix sp.* (willow), *Platanus sp.* (sycamore), and *Ricinus communis* (castor bean). We focused on these three host species, because they are easy to harvest and are present in multiple locations throughout southern California. Our collection sites ranged from Ventura County being the furthest north to San Diego County being the furthest south (Table 1). Multiple infested branches were collected from at least two trees at each location. Infested branches were brought back to UCR and maintained in an insectary room 12 hrs of light and 12 hrs of dark and a relative humidity of 30 to 35 percent. Each branch was cut to fit the glass jar for emergence and both cut ends were waxed to minimize water loss. The glass jar had metal or cloth mesh for ventilation. Larger branches and logs were placed in rearing tents. The dimensions of the tents vary due to the different sizes of the collected logs. Emerging insects were collected daily. Collected specimens were immediately placed in vials with 95 percent ethanol for preservation. Specimens were separated based on their morphotype, location, and log collection date. The number of specimens collected was recorded daily.

3.3.2 DNA extraction and sequencing

For each insect specimen we determined the DNA sequence of part of the mitochondrial cytochrome oxidase *c* subunit I (COI) gene. DNA was extracted using hot sodium hydroxide and Tris, commonly known as the HotSHOT method (Rugman-Jones *et al.*

2020). We chose HotShot method, because it was non-destructive and we could save the insect vouchers to be deposit in the University of California, Riverside Entomology Research Museum later. After the extraction, a polymerase chain reaction (PCR) was used to amplify a portion of the mitochondrial gene (mtDNA) COI for each collected specimen using the LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5' TAAACTTCAGGGTGACCAAAAAATCA-3') barcoding primers (Folmer et al. 1994). PCR was performed in a total of 25 μL reaction consisting 2 μL of DNA extraction material, 1X ThermoPol PCR Buffer (New England Biolabs, Ipswich, Massachusetts), 1 mM MgCl2, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 400 μM dUTP, 20 μg of BSA (New England Biolabs, NEB), 1 U of Taq polymerase (NEB) and forward reverse primers at a concentration of 0.2 μM. The reactions were then placed in a Mastercycler (Eppendorf North America Inc., New York, New York), which was set to denaturation for 2 minutes at 94◦C. This step was followed by five cycles each consisting of 30 seconds at 94◦C, 1 minute 30 seconds at 45◦C and one minute at 72◦C. The reaction was then followed by 35 cycles each consisting of 30 seconds at 94◦C, 1 minute 30 seconds at 51◦C and 1 minute at 72◦C. Subsequently followed by a final extension of 5 minutes at 72◦C. PCR amplification was confirmed using standard agarose gel electrophoresis. If a PCR product did not amplify, then a repeat of DNA extraction from the same specimen and PCR was performed until the sample amplified. PCR products were cleaned using the Wizard PCR Preps DNA purification system (Promega, Madison, Wisconsin) and sequenced in both directions at the Institute for Integrative Genome Biology located at the University of California, Riverside. Sequences were aligned and

trimmed (removing the PCR primers) using Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, Michigan).

3.3.3 Identification of the specimens based on the COI sequence.

The trimmed sequences were then aligned manually. Subsequently the sequences were submitted to the BarCode of life and BLAST identification system in December 2022 (again in March 2023) and identified. If there was no exact match for the sequenced DNA, the closest genus or family that matched the sequenced DNA was assigned.

3.3.4 Specimen habitat and life history analysis

After identifying each specimen, we searched the literature for any habitat and feeding information. After noting the life history of the collected specimens, it was then compared with the life history of shot hole borers in an attempt to assign the insect as a possible competitor (fungal feeder), predator, or parasitoid.

3.4 Result

A total of 2,376 insects were collected, with 51 families identified. Out of all the specimens collected, 157 were sequenced (Table 2, Sequences in Supplementary table 1). Most of the non-sequenced collected insects were shot hole borers. Diptera and Hymenoptera formed the majority of insects that shared the same environment as SHB (36% and 24 % respectively).

From one of our sampling spots (VC2) a larger number of insects emerged from the collected logs than from all other sampling areas combined (total sample 110), with 60% if the specimens belonging to the Diptera, 21% Hymenoptera and 7% Coleoptera (Table 3). From all other locations the combined the sample consisted of 104 specimens with four orders forming the majority with Coleoptera 41%, Diptera 22%, Hymenoptera 19% and Lepidoptera 11% (see table 2 and 3). The collection site VC2 consisted of a wet willow habitat, while most of the remaining sampling sites had less or no standing water.

A minority of the collected and identified specimens are expected to have interactions with the SHBs. 11.2 % were classified as fungal feeders, 14.2% as potential predators, and 7.3% as potential parasitoids (Table 4).

3.5 Discussion

From our data we cannot identify a particular parasitoid or predator that occurs widespread and may substantially affect the shot hole borer populations. Several predators have been identified in our sample that are known predators of Scolytinae, for instance the Monotomidae and Dolichopodidae, however they only formed a small percentage of our sample. Of the parasitoids it is unknown if they would parasitize the beetles, some are known to parasitize weevils, however again their number in our samples is low. Similar studies executed in Florida and Mexico, where the presence of parasitoids was determined in logs infested with invasive ambrosia beetles (Pena et al. 2015; Gómez 2017: Jacobo-Macías et al. 2022). In none of these studies were parasitoids of ambrosia beetles confirmed, although particularly in the Mexican studies parasitoids were identified that are known to parasitize Scolytinae (Gómez 2017: Jacobo-Macías et al. 2022). A problem with our data is twofold, first the identification of some of the specimens is less than precise because no complete match was found for the COI sequence in the existing databases, and secondly because even if a name could be associated with the specimen their life-history is insufficiently known. Future studies on this system should include the analysis of DNA found in the gut of potential predators and parasitoids to unambiguously establish the relationship between a potential natural enemy and the shot hole borers. Is it also possible that the number of potential predators/parasitoids is underestimated in our samples. The collection and transport of logs from the field will have reduced the presence of adult predators in our samples. They could have easily flown away or fallen off the logs when the sample was collected. Then

the only stages of these predators present inside the logs could potentially have been sampled, however only those that would pupate inside the logs could have been included in our sample once they emerged as adults. Those that leave the logs and pupate in the soil were most likely lost in our sampling effort. No soil was provided in our emergence cages for them to pupate in and they may have died unnoticed in these cages. Future sampling should include soil to provide a medium for pupation, together with a regular sifting of the soil to collect pupating insects.

A large fraction of the insects we identified were Diptera many of which were classified as fungal feeders. This may be the result of the habitat in which the logs were collected-willow associated with standing water. So overall, there appears to be little evidence of a large abundance of parasitoids and or predators in our samples, which by themselves could explain the reduction in severity of the Shot hole borer populations. It is clear that we do not know which factors are causing the reduction of the shb populations.

Several population controlling factors have not yet been studied that may explain the reduced shb populations: 1) community resistance, a large number of different competitors, parasitoids and predators that each have a minor influence on the shot hole borer population but together exert control. 2) While in this study we concentrated on arthropods that may regulate shot hole borer populations, in our study, other studies have shown that infestation by phoretic nematodes may result in a reduction of the fitness of the SHBs (Husein, 2023). 3) Finally, studies of bark beetles have shown that sometimes entomopathogenic fungi can reach very high levels in the populations that may result in the decline of the populations (Wegensteiner et al. 2015, Hyblerová et al. 2021).

Determination of the effect of both entomopathogenic fungi and nematodes on the shot hole borers collected from the field is technically difficult, and requires either extensive molecular work or detailed microscopy. 4) Other potential predators are not sampled in our study but may also play some role in the control of the beetle populations, including birds and lizards. In casual field observations fence lizards have been seen to show high activity on trees heavily infested with shothole borers. 4) Trees may become resistant to the growth of the beetle's symbiotic fungi after initial exposure, thus resulting in a decline in suitability of the host trees over time (Guevara-Avendaño, 2019; Biederman et al. 2019).

3.6 Tables

Table 1.1. Collection locality information for Shot-hole Borer infested wood

Location (county)	Location code	Location	Collected date	GPS coordinates	Host plant
LA County	LA1	Whittier Narrows Rec Area	042921	34.034779, -118.056659	*Platanus sp.*
LA County	LA1.1	Whittier Narrows Rec Area	062221	34.034779, -118.056659	*Platanus sp.*
LA County	LA1.2	Whittier Narrows Rec Area	091521	34.034779, -118.056659	*Platanus sp.*
LA County	LA2	Pico Rivera	043021	34.0221600, -118.0546580	*Ricinus communis*
LA County	LA3	Huntington	102021	34.126968, -118.114353	*Salix sp.*
Orange County	OC1	Crystal cove	040521	33.576878, -117.842100	*Salix sp.*
Orange County	OC1.1	Crystal Cove	062221	33.576878, -117.842100	*Salix sp.*
Orange County	OC2	Anaheim	072221	33.8744233, -117.7501016	*Salix sp.*
Orange County	OC2.1	Anaheim	121321	33.8744233, -117.7501016	*Salix sp.*
Riverside County	RC1	Riverside	072221	33.900887, -117.423118	*Ricinus communis*
Riverside County	RC2	Anza park	051021	33.965033, -117.421854	*Ricinus communis*
Riverside County	RC3	UC Riverside	080421	33.963635, -117.340383	*Quercus sp.*
Riverside County	RC4	Norco	072221	33.946905, -117.553553	*Salix sp.*
San Diego County	SD1	Carlsbad	070921	33.1816216, -117.3237414	*Salix sp.*

San Diego County	SD2	Tijuana Valley	081921	32.556605, -117.089303	*Salix sp.*
Ventura County	VC1	Santa Clara River	043021	34.3627430, -118.9962850	*Salix sp.*
Ventura County	VC2	Oak View	052421	34.3961460, -119.3162402	*Salix sp.*
Ventura County	VC3	Oak View	062321	34.3961460, -119.3162402	*Salix sp.*
Ventura County	VC4	Oak View	091421	34.3961460, -119.3162402	*Salix sp.*
Ventura County	VC5	Oak View	121321	34.3961460, -119.3162402	*Salix sp.*

Table 2. Insects and arachnids that shared the same environment as SHB and their collection location and feeding classification with respect to potential interactions with SHB life stages and fungal food source based on literature search: Predator (Pred), Parasitoid (Par), Fungal feeder (FF), no interaction expected (No)

Family	**Species**	**COI sequence identifier**	**Location**	**Food**	**Potential interactions with SHBs**	**# of specimens collected**
Arachnida: Araneae						
Theridiidae	*Platnickina alabamensis*	ICP22068	VC2	Other insects (58)	Pred	1
Hahniidae	*Cicurina* sp.	ICP22071	VC2	Other insects	Pred	1
Arachnida: Mesostigmata						
Ascidae	*Proctolaelaps ?* sp *(Acari)*	PR21533	LA2	Predators of other mites (54)	?	1
Arachnida: Pseudoscorpiones						
Cheliferidae	sp.	ICP22070	VC2	Other insects	Pred	1
Blattodea						
Kalotermitidae	*Incisitermes minor*	ICP22014	VC2	Woody plant material (59)	No	1
Coleoptera						
Cerambycidae	*Saperda horni*	Identified by museum specialist	RC1	Larvae and adult mostly feed on plants, few preys(4)	No	1

Cerambycidae	Malacopterus tenellus	Identified by museum specialist	VC2	Larvae and adult mostly feed on plants, few preys(4)	No	1
Colydiidae	*Lasconotus* sp.	PR21646 PR21647 PR21648	LA1.1	Predator of *Ips. spp.* (another bark beetle) (65)	Pred	3
Cucujidae	*Cryptolestes punctatus*	PR21637 PR21641 PR21645 PR21653 PR21654 PR21665	OC1.1	Not much is known on the biology. Frequently found under barks or wood material (63)	No	6
Curculionidae	Pentarthrum sp.	PR21655 PR21659	VC3	Fungal feeder in trees (64)	FF	2
Curculionidae	sp. (Non-SHB)	PR21655 PR21656 PR21657 PR21658 PR21659 PR21661 PR21668	VC3	Plant and fungal materials	No	7
Dermestidae	*Megatoma* sp.	ICP22074 ICP22078	VC1	Not much life history is known (59) Likely to feed on animal debris	No	3

Laemophloeidae	*Leptophloeus juniperi*	PR21637 PR21641 PR21642 PR21644 PR21662 PR21663	OC1.1	Predators of other Bark beetles (66).	Pred	6
Leiodidae	sp.	PR21638 PR21646	LA1.2	Fungi and carrion	FF	2
Melyridae	sp.	ICP22152	RC1	Larvae feed on fungi, detritus, and small arthropods (5)	Pred	3
Monotomidae	sp.	ICP22087	OC1	Predator (1)	Pred	1
Monotomidae	*Bactridium* sp.	PR21666	OC2.1			1
Ptinidae	*Ptilinus basalis*	ICP22134	VC2	Decaying wood (2)	No	7
Scarabaeidae	*Cleptocaccobius convexifrons*	PR21647 PR21648 PR21652 PR21654 PR21665	LA1.1	No much life history information was found. Likely fungus or animal debris	FF?	5
Staphylinidae	Aleocharinae sp.	PR21639	VC4	Diptera predator (67)	No	1
Staphylinidae	*Neolispinus* sp.	PR21660	VC5	Not much life history is found	?	1
Staphylinidae	*Pseudopsis montoraria*	PR21660	VC5	Not much life history is	?	1

				found		
Diptera						
Anthomyiidae	*Coenosopsia* sp.	ICP22110	VC2	Not much is known about it most likely on fungus for larvae(8)	?	2
Anthomyiidae	sp.	ICP22143	VC2	Not much is known about it most likely on fungus for larvae(8)	?	1
Cecidomyiidae	sp.	ICP22016 ICP22019 ICP22106 ICP22147 ICP22153	VC2	Immature in plant galls, adults short lived and likely won't feed. (17)	No	10
Cecidomyiidae	sp.	ICP22108 ICP22122 ICP22140 ICP22157	RC4	Immature in plant galls, adults short lived and likely won't feed. (17)	No	3
Cecidomyiidae	sp.	ICP22148	RC1	Immature in plant galls, adults short lived and likely	No	3

				won't feed. (17)		
Cecidomyiidae	sp.	ICP22114	LA3	Immature in plant galls, adults short lived and likely won't feed. (17)	No	2
Ceratopogonidae	*Forcipomyia* sp.	ICP22105, ICP22120, ICP22154, ICP22156	VC2	Larvae are gregarious, feed on fungi and decay materials, Adult on insect or mammal body fluids (9)	FF	6
Ceratopogonidae	*Forcipomyia* sp.	ICP22095 ICP22117	LA3	Larvae are gregarious, feed on fungi and decaying materials, Adult on insect or mammal body fluids (9)	FF	2
Ceratopogonidae	Forcipomyiinae sp.	ICP22097	VC2	Larvae aquatic and adult feed on small Nematocera, (15) pollen,	No	1

				and nectar (16).		
Ceratopogonidae	sp.	ICP22007 ICP22009 ICP22017 ICP22024 ICP22046 ICP22069 ICP22102 ICP22104 ICP22115 ICP22160	VC2	Larvae aquatic and adult feed on small Nematocera, (15) pollen, and nectar (16).	No	11
Ceratopogonidae	sp.	ICP22004 ICP22116	SD2	Larvae aquatic and adult feed on small Nematocera, (15) pollen, and nectar (16).	No	2
Chloropidae	*Oscinellinae* sp.	ICP22036	VC2	some phytophagous and few are gall inducers and predaceous (12)	No	6
Chloropidae	*Oscinellinae* sp.	ICP22141	RC4	some phytophagous and few are gall inducers and predaceous (12)	No	1

Mycetophilidae	sp.	ICP22130	OC1	Fungivores (18)	FF	1
Drosophilidae	*Rhinoleucophenga punctulata*	ICP22092	VC2	Not much life history is known. Likely to be larval predators of Sternorrhyncha (scale inscets) (61)	No	1
Dolichopodidae	*Gymnopternus* sp.	ICP22088	VC2	Not much life history is known	?	1
Odiniidae	*Odinia?* sp. *[BOLD: AAG4783]*	PR21284	VC2	Feed on other beetle species's pupae. (11)	Pred	1
Odiniidae	sp.	PR21289	VC2	Feed on other beetle species's pupae. (11)	Pred	1
Odiniidae	sp.	ICP22162	SD2	Feed on other beetle species's pupae. (11)	Pred	1
Periscelididae	sp.	PR21278	OC1	Family usually associated with sap fluxes	No	1

				from deciduous trees (45)		
Sciaridae	*Bradysia tilicola*	ICP22086	VC1	Feed on fungi(7)	FF	3
Sciaridae	*Claustropyga acanthostyla*	ICP22015 ICP22101 ICP22103 ICP22107	VC2	Not enough studies, likely to feed on fungi	?	15
Sciaridae	*Claustropyga* sp.	ICP22057	VC2	Not enough studies, likely to feed on fungi	?	1
Sciaridae	sp.	ICP22012 ICP22111 ICP22112 ICP22118 ICP22123	VC2	Feed on fungi (10)	FF	5
Sphaeroceridae	*Pullimosina pullula*	ICP22034	VC2	Decaying vegetation (13)	No	3
Sphaeroceridae	*Pullimosina pullula*	ICP22126	OC1	Decaying vegetation (13)	No	3
Syrphidae	*Brachypalpus oarus*	ICP22109	VC2	Decaying matters (6)	No	3
Tachinidae	*Senometopia pollinosa*	PR21667	OC2.1	Not much Life history is know	?	1
Tephritidae	*Dacus telfaireae*	ICP22033	VC2	Plant materials (67)	No	1
Ulidiidae	*Euxesta pechumani*	ICP22013	VC2	Animal fecal matter (68)	No	1

Hemiptera						
Anthocoridae	*Anthocoris musculus*	ICP22006	VC2	Plant material (69)	No	1
Hymenoptera						
Aphelinidae	*Coccophagus rusti*	PR21530	SD2	Parasitoid of coffee green scales (31)	No	1
Aphelinidae	*Coccophagus rusti*	PR21541	VC2	Parasitoid of coffee green scales (31)	No	1
Bethylidae	*Sclerodermus guani/sichuanensis - [BOLD: private-California]*	PR21275 PR21280	OC1	Parasitoid of some Cerambicidae (47) and Tenebrionidae (48)	Par	2
Bethylidae	*Sclerodermus sichuanensis*	ICP22125	OC1	Parasitoid of Cerambicidae (49)	Par	1
Braconidae	*Atanycolus* sp. *BIOUG10906-B04*	PR21547	RC3	Parasitoid of Buprestidae (26), Curculionidae, Cerambycidae, and Scolytidae(27)	Par?	1

Braconidae	*Dolichogenidea* sp. *[BOLD: ACA6623]*	PR21534	OC2	parasitoid of Lepidoptera (33)(34)	No	1
Braconidae	*Hecabolus* sp.*1 [BOLD: private-California]*	PR21286, PR21287, PR21288, PR21544	VC2	ectoparasitoid of larvae in families Anobiidae, Cerambycidae, Chrysomelida, Curculionidae, and Ptinidae (40)	Par?	4
Braconidae	*Mirax* sp. *[BOLD: ACM1203]*	PR21527	SD2	Parasitoid of Lepidoptera (42),(43)	No	1
Ceraphronidae	*Aphanogmus* sp.	PR21540	VC2	Hemipteran, Dipteran parasitoid (21) (22)	No	1
Ceraphronidae	*Aphanogmus* sp.	PR21277	OC1	Hemipteran, Dipteran parasitoid (21) (22)	No	1
Encyrtidae	*Blepyrus* sp.	PR21282	VC2	Hemipteran parasitoid (28)	No	1
Encyrtidae	*Blepyrus* sp.	PR21290	LA2	Hemipteran parasitoid (28)	No	1

Encyrtidae	*Cheiloneurus* sp.	PR21545	VC2	hyperparasitoid of various encyrtid primary parasites (30)	No	1
Encyrtidae	*Comperiella bifasciata*	PR21523	OC1	Scale parasitoid (32)	No	2
Encyrtidae	*Lamennaisia* sp.	PR21281 PR21543	VC2	Coccoidea Noyes Chalcidoidea Database	No	2
Encyrtidae	*Lamennaisia* sp.	PR21529	SD2	Coccoidea Noyes Chalcidoidea Database	No	1
Encyrtidae	*Metaphycus anneckei*	IC22182	VC2	Parasitoid of black scale (41)	No	1
Eulophidae	*Chrysocharis clarkae*	PR21532	SD2	Hemipteran parasitoid (20)	No	1
Eulophidae	*Aprostocetus* sp.	PR21531	SD2	Egg parasitoid of some Curculionidae (23), Cerambycidae (24) and Blattodea (25)	Par?	1

Eulophidae	*Aprostocetus* sp.1	PR21535, PR21542	VC2	Egg parasitoid of some Curculionidae (23), Cerambycidae (24) and Blattodea (25)	Par?	2
Eulophidae	*Elasmus* sp.1 - *[BOLD: ACM1170]*	PR21271 PR21272 PR21273 PR21274	OC1	Parasitoid of other Hymenoptera (35), Lepidoptera (36)	No	1
Eulophidae	*Elasmus* sp.1 - *[BOLD: ACM1170]*	PR21548	RC3	Parasitoid of other Hymenoptera (35), Lepidoptera (36)	No	1
Eulophidae	*Euderus albitarsis* - *[BOLD : AAG7982]*	PR21521	N/A	Parasitoid of cabbage seedpod weevil (Coleoptera: Curculionidae) (37)	Par?	1
Eurytomidae	*Eurytoma* sp.	PR21283	VC2	Gall wasp (38) (39)	No	1
Eupelmidae	*Brasema* sp.	PR21276	OC1	Parasitoid of Coleoptera Hemiptera, and Diptera (29)	Par	1

Formicidae	*Linepithema humile*	ICP22028	VC2	Forager, and sometimes predator of other insects.	Pred	6
Formicidae	*Tapinoma* sp.	ICP22018	VC2	Forager, and sometimes predator of other insects.	Pred	1
Formicidae	*Linepithema humile*	ICP22158	LA3	Forager, and sometimes predator of other insects.	Pred	1
Pteromalidae	*Neocalosoter?* sp.	PR20565	SD2	Parasitoid of walnut twig beetle (44)	Par	1
Pteromalidae	sp.	ICP22065	VC2	Wide range of insect host.	Par?	1
Scelionidae	*Telenomus laricis group [BOLD: ACJ9786]*	PR21537	VC2	Egg parasitoid of Heteropteran (50)	Par?	1
Lepidoptera						
Ascidae	*Proctolaelaps ?* sp *(Acari)*	PR21533	LA2	Predators of other mites (54)	?	1
Batrachedridae	*Batrachedra pinicolella*	ICP22131	OC1	Needle miners of pine (52)	No	3

Pyralidae	*Ephestiodes gilvescentella*	ICP22100	Durfee	Dried fruit pest (53)	No	1
Tineidae	*Xylesthia albicans*	ICP22089 ICP22090 ICP22136 ICP22137	LA1	No life history was found	?	3
Tineidae	*Oinophilia v-flava*	ICP22003 ICP22054 ICP22079	RC1	No life history was found	?	5
Tineidae	*Oinophilia v-flava*	ICP22077	VC2	No life history was found	?	1
Tineidae	*Opogona arizonensis*	ICP22073	VC2	Not much life history was found, Likely to feed on plant material like it's sister species (70)	No	3
Psocodea						
	sp.	ICP22032, ICP22047	VC2	Likely fungi or other decaying plant matter	FF	1
Psocidae	*Trichadenotecnum* sp.	ICP22053, ICP22060, ICP22076, ICP22145	VC2	Likely fungi or other decay plant matter	FF	2
Trogiidae	*Cerobasis guestfalica*	ICP22072	VC2	Likely to be	No	1

				feeding on woody material		
Thysanaptera						
Phlaeothripidae	*Haplothrips* sp.	ICP22129	OC1	Feed on pollen	Pred?	4

1) Meurisse et al. 2008, (2) Niemiec 1995 (3) Gosik et al. 2019 (4) Haack et al. 2017 5) Arnet et al. 2002, (6)Thompson and Vockeroth 1989, (7) Kecskeméti et al. 2019, (8) Nihei and Carvalho 2004, (9) Saunders 2009, (10) Cloonan et al. 2016, (11) Gaimar 2010, (12) Riccardi 2016, (13) Marshall 1986, (14) Marshall and Cui 2005, (15) Grogan and Wirth 1977, (16) Grogan 2020, (17) Tokuda and Yukawa 2005, (18) Gagné 1975, (19) Grimes and Cone 1985, (20) Mujica and Kroschel 2011, (21) Youssef et al. 2022, (22) Matsuo et al. 2016, (23) Ulmer et al. 2006, (24) Wang et al. 2021, (25) Tee and Lee 2015, (26) Duan 2016, (27) Urano and Hijii 1991, (28) Silva et al. 2017, (29) Marchiori 2021, (30) Weseloh 1969, (31) Murphy 1991, (32) Flanders 1944, (33) Badrulisham et al. 2022, (34) Aigbedion-Atalor et al. 2020, (35) Reed and Vinson 1979, (36) Ramachandra and Cherian 1927, (37) Dosdall et al. 2008, (38) McDaniel and Boe 1991, (39) Wikler et al. 1996, (40) Castañeda-Osorio et al. 2022, (41) Daane et al. 2000, (42) Özgen et al. 2012, (43) Gallardo-Covas 1992, (44) Bosio and Cooke-McEwen 2018, (45) Mathis and Rung 2011, (46) Coronado Blanco et al. 1998, (47) Wu et al. 2017, (48) Wu et al. 2013, (49) Liu et al. 2011, (50) Rouault et al. 2007, (51) Yee et al. 2021, (52) Maier 2005, (53) Simmons and Nelson 1975, (54) Gondim et al. 2007, (55) Sommerman 1943, (56) Brues 1927, (58) Guarisco (2018), (59) Cabrera and Rust 1998, (60) Kadej and Hava 2016, (61) Culik and Ventura 2009

Table 3. Composition of samples by number of specimens belonging to different orders taken at location VC2 and all other locations combined

Location VC2			All other locations combined		
Order	# of specimens	Percentage of sample	Order	# of specimens	Percentage of sample
Blattodea	1	0.9			
Coleoptera	8	7.3	Coleoptera	43	41.3
Diptera	66	60.0	Diptera	23	22.1
Hemiptera	1	0.9			
Hymenoptera	23	20.9	Hymenoptera	20	19.2
Lepidoptera	4	3.6	Lepidoptera	12	11.5
Pseudoscorpiones	1	0.9	Mesostigmata	2	1.9
Psocodea	4	3.6	Thysanaptera	4	3.8
Araneae	2	1.8			
Total	110			104	

Table 4. Composition of samples by number of specimens belonging to different classification with regards to potential interaction with ambrosia beetles based on the classification into Predator, Parasitoid, Fungal Feeder, unknown relationship based on literature (see text), taken at location VC2 and all other locations combined

	VC2		All other locations combined	
Potential interaction with Ambrosia beetles	# of specimens	Percentage	# of specimens	Percentage
Fungal feeder (Competitor)	10	9.1	15	14.4
Predator	12	10.9	20	19.2
Parasitoid	8	7.3	8	7.7
No expected interaction	57	51.8	47	45.2
Unknown –unlikely to interact	23	20.9	14	13.5
Total	110		104	

4. Conclusions

We find that it was not possible to correctly identify the beetles using morphological characters. Only molecular techniques based on the COI sequence could correctly identify the taxa in the *E. fornicatus* species complex. Using morphological characters alone results in a less than 50% correct identification. Environmental factor such as different plant hosts influences size of the characters used in the morphometric identification. There are other factors such as humidity, temperature, precipitation, tree health that were not examined in this chapter, but may also affect the size of the beetles.

From the host samples that we collected, we sequenced all the insects that emerged out from the plant material. We did not find any specific natural enemies of SHB. There are recorded insects that feed on other Curculionidae, however, the connection with SHB is unknown. The cause of the sudden decline of SHB populations is still unknown. It could be a result of the boom-bust dynamic in which the beetle population had a drastic population incline followed by a drastic incline. The sudden increase in population numbers would have been the result in the establishment of the pest when there were no active controls present (Strayer et al. 2017). KSHB population reached the peak during 2015 and 2016 (Boland and Uyeda, 2020) and suddenly decreased in 2019. The cause of the decline is as yet not known, no clear natural insect parasitoid or predator was identified in our study. We suggest that future research into the cause of the beetle decline should include the sampling of both nematodes and entomopathogenic fungi to get a more complete insight into the natural enemies of the shot hole borers.

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