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ORIGINAL ARTICLE

Comparative morphometric and chemical analyses of phenotypes of two invasive ambrosia beetles (*Euwallacea* spp.) in the United States

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Abstract The polyphagous shot hole borer (PSHB), *Euwallacea* sp., was first detected in 2003 in Los Angeles County, California, USA. Recently, this invasive species has become a major pest of many hardwood trees in urban and wildland forests throughout southern California. PSHB is nearly identical in morphology and life history to the tea shot hole borer (TSHB), *Euwallacea fornicatus*, an invasive pest of hardwoods in Florida, USA and many other parts of the world. However, molecular studies have suggested that the taxa are different species. We conducted morphometric and chemical analyses of the phenotypes of *Euwallacea* sp. collected in southern California (Los Angeles County) and *E. fornicatus* collected in Florida (Miami-Dade County). Our analyses indicated that PSHB has 3 larval instars. The third larval instar was separated from the first 2 instars by head capsule width with 0 probability of misclassification. The body length, head width, and pronotal width of PSHB adult males were significantly less than those of females. Head width and pronotal width of female PSHB were significantly less than those of female TSHB. In contrast, body length, and ratio of body length to pronotal width of female PSHB were significantly greater than those of female TSHB. However, females of these 2 species could not be separated completely by these 4 measurements because of the overlapping ranges. Cuticular hydrocarbons detected in both species were exclusively alkanes (i.e., *n*-alkanes, monomethylalkanes, dimethylalkanes, and trimethylalkanes). Cuticular hydrocarbon profiles of PSHB males and females were similar, but they both differed from that of TSHB females. Cuticular hydrocarbons of PSHB were predominantly internally branched dimethylalkanes with backbones of 31 and 33 carbons, whereas cuticular hydrocarbons of TSHB females were dominated by internally branched monomethylalkanes and dimethylalkanes with backbones of 28 and 29 carbons. Multiple compounds within these classes appear to be diagnostic for PSHB and TSHB, respectively.

Key words cuticular hydrocarbons; *Euwallacea fornicatus*; larval instars; polyphagous shot hole borer; Scolytidae; tea shot hole borer

Introduction

The polyphagous shot hole borer (PSHB), *Euwallacea* sp. (Coleoptera: Scolytidae, *sensu* Bright, 2014) (Coleman *et al.*, 2013), was discovered on 30 May 2003 in Whittier Narrows Recreational Area, near South El Monte, Los Angeles County, California, USA (Seybold

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et al., 2016), and as of 2016 has spread to 4 neighboring or nearby counties (Orange, Riverside, San Bernardino, and Ventura). PSHB in California was first reported erroneously as tea shot hole borer (TSHB), *Euwallacea fornicatus* (Eichhoff), but subsequent sequencing of nuclear and mitochondrial DNA indicated that the introduced population in California was likely a separate species (Eskalen *et al.*, 2013). The introduced population (or populations) of PSHB likely originated from southeastern Asia (http://caforestpestcouncil.org/wp-content/uploads/2008/07/Polyphagous-Shot-Hole-Borer.pdf, accessed 1/1/2016). In 2013, an established population of another closely related species or subspecies (likely from Japan or China [Taiwan island] and referred to provisionally as the Kuroshio shot hole borer [KSHB]) was also detected in southern California (San Diego County) (http://ucanr.maps.arcgis.com/apps/Viewer/index.html?appid=3446e311c5bd434eabae98937f085c80, accessed 1/1/2016).

Limiting further spread of PSHB in California by preventing movement of infested wood from the invaded areas and chipping infested wood on site are 2 currently recommended management options for this invader (http://ucanr.edu/sites/socaloakpests/Polyphagous_Shot_Hole_Borer/, accessed 1/1/2016). Insecticide trials for PSHB have not been reported. However, effective pest management programs involving insecticides for control typically target the adult and larval stages. Thus, differentiating the developmental stages of the target insects can be critical. Toxicity of insecticides to an insect has long been known to decrease with the age of the insect (McPherson *et al.*, 1956; Rock *et al.*, 1961; Eldefrawi *et al.*, 1964; Yu, 1983; U.S. Department of Agriculture, 1989; Bouvier *et al.*, 2002). Identifying the developmental stages of target pests is also critical for the success of biological control programs that utilize larval parasitoids (Hanks *et al.*, 2001; Beckage *et al.*, 2003; Chen, 2007). For example, *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae), the primary mortality agent for the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), and tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Noctuidae) (Beckage *et al.*, 2003), in the southeastern USA (Chen & Ruberson, 2008), develops faster and survives more successfully in early instar *S. exigua* (Chen, 2007).

The number of larval instars of laboratory-reared insects can be determined by the number of molts (i.e., the number of cast exuviae per individual). However, this is not possible with field-collected larvae because their development cannot be monitored as precisely as laboratory-reared insects. Maximum likelihood analysis has been applied recently for instar determination (Flaherty *et al.*, 2012; Bleiker & Régnière, 2014), but another widely used method is based on frequency distribution (Dyar, 1890; Logan *et al.*, 1998; Dallara *et al.*, 2012; Chen & Seybold, 2013). The latter method has proven to be robust even when the assumption of normality of the distribution of head capsule widths of each instar is violated (Chen & Seybold, 2013).

The numbers of larval instars of many bark and ambrosia beetles (Coleoptera: Scolytidae) (*sensu* Bright, 2014) have been determined by using the frequency distribution method (see Lekander, 1968 and Dallara *et al.*, 2012 for reviews). Although some species might undergo 2 or 6 larval instars, most species have between 3 and 5 larval instars (Lekander, 1968; Dallara *et al.*, 2012). PSHB and TSHB belong to the tribe Xyleborini, and two other species in this tribe have 3 larval instars (reviewed in Dallara *et al.*, 2012). A few studies have described life cycles and characterized infestations of TSHB in the native Asian range (Kumar *et al.*, 2011; Li *et al.*, 2014, 2015), and both 3 (Kumar et *al.*, 2011, http://entomology.ifas.ufl.edu/creatures/trees/beetles/tea_shot_hole_borer.htm, accessed 1/1/2016), and 5 (Li *et al.*, 2014) larval instars have been reported. It was not clear how the number of larval instars was determined in these studies. There have been no reports on the number of larval instars of PSHB; in this paper we determined the number of PSHB larval instars by using the frequency distribution method.

PSHB is haplodiploid (i.e., males develop from unfertilized eggs and are haploid, whereas females develop from fertilized eggs and are diploid). Females (Fig. 1A) and flightless males (Fig. 1B) can be distinguished by body color, length, and presence/absence of the pair of membranous hind wings. PSHB females (1.80–2.50 mm) are longer than males (1.50–1.67 mm), and they are also darker in color than males (http://cisr.ucr.edu/polyphagous_shot_hole_borer.html, accessed 1/1/2016). Because it has only recently been suggested as a potentially new species, other characters separating the sexes of PSHB, and PSHB from TSHB, are unknown. Males and females of TSHB can be distinguished by body length, elytral length, pronotal sizes, morphology of the elytral declivity, etc. (Rabaglia *et al.*, 2006). In an attempt to study other morphological characters that can be used to distinguish PSHB males from females, we measured body length (apex of elytra to anterior margin of dorsal pronotum), maximum pronotal width, and maximum head width. Furthermore, although Kumar *et al.* (2011) reported body measurements of TSHB larvae and adults, these authors did not define which

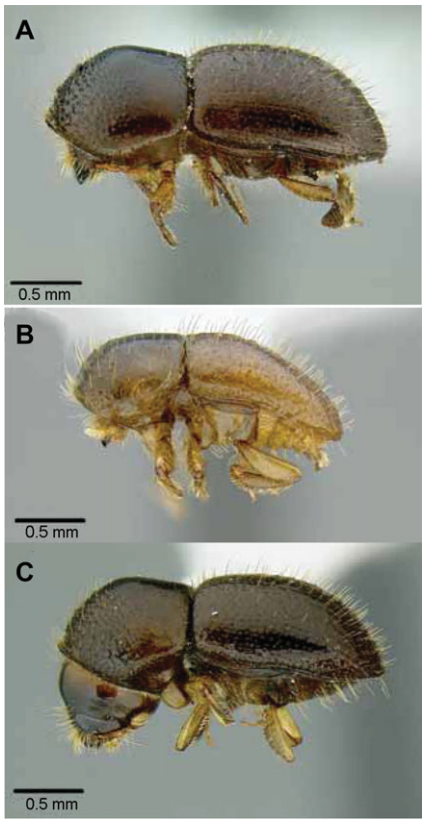

Fig. 1 Automontage photographs of adult *Euwallacea* sp. female (A), male (B), and *Euwallacea fornicatus* female (C) (S.M. Hishinuma, photos). High-resolution digital images were captured by using a JVC KY-F75U digital video camera (JVC Professional Products Company, Wayne, NJ, USA) attached to a Leica MZ16 stereomicroscope (Meyer Instruments, Inc., Houston, TX, USA). All images were compiled by using Syncroscopy Auto-Montage (Synoptics Ltd., Cambridge, UK).

body parts were measured. Thus, we measured and compared body length, maximum pronotal width, and maximum head width of male and female PSHB and female TSHB.

Cuticular hydrocarbons play a variety of intra- and interspecific roles in insect societies and communities including task decision (Greene & Gordon, 2003), nestmate recognition (Martin & Drijfhout, 2009; Sturgis & Gordon, 2012), and mate recognition (Ferveur, 2005). Some predators (e.g., the salticid spider, *Cosmophasis bitaeniata*) mimic cuticular hydrocarbons of their prey for access to prey colonies (Elgar & Allan, 2004). Cuticular hydrocarbon profiles have proven useful in separating different species, in particular for those closely related species that are extremely similar or indistinguishable morphologically (Page *et al.*, 1990a,b, 1997; Kaib *et al.*, 1991; Haverty *et al.*, 2003; Martin *et al.*, 2008; Nelson *et al.*, 2008; Lim & Forschler, 2012). We collected and analyzed cuticular hydrocarbons from adult PSHB and TSHB to determine their utility in supplementing genetic data for species separation.

Materials and methods

Collection of polyphagous shot hole borer larvae for instar determination

PSHB larvae from Los Angeles County, California were collected from logs used in a PSHB host range study, which will be reported elsewhere. In that study, PSHB females that had emerged from field-collected logs of boxelder, *Acer negundo* L. (collected on 5 May 2014 at 1920 N. Santa Anita Avenue, Arcadia, California; GPS: N 34.167349°, W 118.031810°, elev. 273 m) and castor bean, *Ricinus communis* L. (collected on 5 May 2014 on the Angeles National Forest, Los Angeles Ranger District, Chantry Flat Road; GPS: N 34.18396°, W 118.02540° elev. 585 m) were constrained individually in gel capsules by using #2 insect pins (Catalog #1208B2, BioQuip Products, Rancho Dominguez, CA, USA) and forced to feed on small diameter (5–10 cm) logs from 25 tree species collected from California, Louisiana, and New Mexico, USA (Table 1). Five to six females were introduced in each log from each tree species. Six to seven logs (all from different source trees) per tree species were tested. As a consequence, the number of test females per tree species ranged from 30 to 42. Logs were cut into small pieces and sectioned longitudinally approximately 6 weeks later. We waited this period of time because the estimated generation time for TSHB is 40 d (Gadd, 1941; Kumar *et al.*, 2011), and by waiting 42 d we anticipated sampling from all possible larval instars. An estimate of the generation time of PSHB under laboratory conditions was not available to us. Due to the time-consuming collection process, larvae from randomly chosen host tree species and sources were collected for instar determination. In the end, a total of 303 larvae were collected from American sycamore, boxelder, castor bean, English walnut, quaking aspen, red willow, and an unidentified species of willow as secondary hosts in this study. Since we were only interested in collecting a sufficient number of larvae to represent the various instars, the number of larvae collected from each secondary host species was not recorded. Larval head capsule maximum width was measured to the nearest 0.01 mm with a Zeiss Stemi 2000 Stereomicroscope (Fisher Scientific, Atlanta, GA, USA) at 50× with an ocular micrometer. Larvae were stored and measured in 70% ethanol.

Table 1 List of tree species tested as potential hosts for polyphagous shot hole borer, *Euwallacea* sp. (Coleoptera: Scolytidae).

California	Louisiana	New Mexico
Big leaf maple, *Acer macrophyllum* Pursh	American sycamore, *Platanus occidentalis* L.	Boxelder, *Acer negundo* L.
Boxelder, *Acer negundo californicum* (Torr. & A. Gray) Sarg.	Black willow, *Salix nigra* Marshall	Gambel oak, *Quercus gambelii* Nutt.
California bay laurel, *Umbellularia californica* (Hook. & Arn.) Nutt.	Red maple, *Acer rubrum* L.	Mountain maple, *Acer glabrum* Torr.
California ash, *Fraxinus dipetala* Hook. & Arn.	Southern red oak, *Quercus falcata* Michx.	Narrow leaf cottonwood, *Populus angustifolia* James
California black oak, *Quercus kelloggii* Newb.		Quaking aspen, *Populus tremuloides* Michx.
California sycamore, *Platanus racemosa* Nutt.		Unidentified willow, *Salix* sp.
Canyon live oak, *Quercus chrysolepis* (Liebm.)		
Castor bean, *Ricinus communis* L.		
Coast live oak, *Quercus agrifolia* Née		
English walnut, *Juglans regia* L.		
Fremont's cottonwood, *Populus fremontii* S. Watson		
Interior live oak, *Quercus wislizeni* A. DC.		
Red willow, *Salix laevigata* Bebb		
Southern California black walnut, *Juglans californica* S. Watson[†]		
White alder, *Alnus rhombifolia* Nutt.		

[†]We note that Hishinuma *et al.* (2016) reported this species as a host of PSHB from field collected material.

Instar determination for polyphagous shot hole borer larvae

Because larvae of TSHB were not available, instar determination for TSHB was not conducted. The method for determination of the number of PSHB larval instars followed Chen and Seybold (2013), which is a modification of the methods of Beaver and Sanderson (1989), McClellan and Logan (1994), and Logan *et al.* (1998). Using cast head capsules of beet armyworm, *S. exigua*, Chen and Seybold (2013) verified that the frequency distribution method for larval instar determination was robust. Briefly, frequency distributions of head capsule widths of the 303 larvae based on 3 different histogram width classes were constructed (PROC UNIVARIATE) (SAS Institute, 2010). Kernel density estimation, a nonparametric technique to estimate the probability density function of a random variable with a Gaussian density, was used to determine the number of peaks, each of which represents 1 instar (k). The entire head capsule data set was then separated into subsets (i.e., instars) based on the minima in the kernel density curve. Means ($\bar{x}_i$) and variances (s_i^2) of each subset were computed. Parameters (i.e., a_i, b_i, and c_i) of the Gaussian density curve of each subset were optimized by using Equation (1) with the nonlinear least squares procedure (PROC NLIN) (SAS Institute, 2010).

$$y_i = a_i e^{-b_i(x-c_i)^2},\ i = \text{instar}1, 2, ..., k, \qquad (1)$$

where y_i is the frequency of each width class, x is the head capsule width, a_i is a scaling parameter, $b_i = 1/(2{s_i}^2)$, and c_i is the mean head capsule width of each subset. The initial estimates of a_i, b_i, and c_i were counts of the most frequent width class, $1/(2{s_i}^2)$, and $\bar{x}_i$, respectively. The estimates of the initial nonlinear least squares parameters, a_i, b_i, and c_i, from Equation (1) were further simultaneously fitted to equation (2) to obtain final nonlinear least squares estimates (PROC NLIN).

$$h_i = \sum\nolimits_i^k a_i e^{-b_i(x-c_i)^2}, \qquad (2)$$

where h_i are the counts of the head capsule width classes for the *i*th instar. The final nonlinear least squares parameters b_i and c_i were then substituted into equation (3) and misclassification probabilities were computed from the intersections between the frequency distributions of the instars.

$$f_i = \frac{1}{\sigma_i \sqrt{2\pi}} e^{-\frac{(x-u_i)^2}{2{\sigma_i}^2}}, \quad (3)$$

where ${\sigma_i}^2 = 1/2b_i$, and u_i is the mean head capsule width of each subset. The misclassification probabilities of classifying instar *i* to $i + 1$ and instar *i* to $i - 1$ were calculated by using 2 methods. Theoretical misclassification probabilities were obtained by solving Equations (4) and (5). The intersection points (l_i) were visually determined from the distributions.

$$P_{(i \text{ to } i+1)} = \int_{l_i}^{\infty} f_i dx, \quad (4)$$

$$P_{(i \text{ to } i-1)} = \int_{-\infty}^{l_i} f_i dx. \quad (5)$$

Brooks–Dyar growth ratios (Dyar, 1890) (ratio of consecutive instar head capsule widths) are frequently used to determine the geometric growth of insect head capsule size, and a linear relation between the natural log of the mean head capsule width for each instar against the corresponding instar number indicates that no intermediate larval instars were overlooked (Daly, 1985; Logan *et al.*, 1998). Brooks–Dyar growth ratios were calculated and the natural log of mean head capsule width for each instar of PSHB was regressed against the corresponding instar number (SigmaPlot 12.0; Systat Software, Inc., San Jose, CA, USA).

Measurements of polyphagous shot hole borer and tea shot hole borer adults

PSHB: A total of 3268 adult PSHB females and 249 males were collected from the above-mentioned host-range study. Males and females were separated by their wing characteristics (i.e., males lacked the pair of membranous hind wings). Adults used for morphometric analyses included brood produced in the above-mentioned host-range study, and adults that emerged from the field-collected boxelder and castor bean logs, but not parental females that were used in the host-range study. Sixty-five males and 100 females were selected randomly from the pool for measurement of body length (dorsal anterior edge of pronotum to apex of elytra) and maximum pronotal width, by using the technique described above for larvae, with the exception that adults were stored and measured dry. Thirty-five males and 35 females were selected randomly for measurement of maximum head width. The differences in body length, pronotal width, and head width between male and female beetles were analyzed by the nonparametric Kruskal–Wallis test (PROC NPAR1WAY in SAS) because the measurements were not normally distributed (Kolmogorov–Smirnov *D* Test). Variance of data was checked with Levene's test. Significance level in all tests was $\alpha = 0.05$.

TSHB: Forty-three (43) females were collected from a colony maintained in a laboratory at the University of Florida. The original beetles for the colony were collected from an avocado grove in Homestead, Miami-Dade County, Florida, USA (GPS: N 25.59458°, W 80.48238°). Forty-one of these were used for morphometric analyses, whereas 43 were used for cuticular hydrocarbon analyses (see below). Insects were extracted for cuticular hydrocarbons and frozen prior to measurement. Maximum head width, body length, and maximum pronotal width were measured as described above. Measurements of TSHB males were not made due to the paucity of males in the laboratory colony.

PSHB body length was compared to the corresponding measurement of TSHB by an independent *t*-test (PROC TTEST in SAS) because the data followed a normal distribution. Maximum head width, maximum pronotal width, and body length to pronotal width ratio of PSHB were compared to the respective measurements of TSHB by the Kruskal–Wallis test (PROC NPAR1WAY in SAS) because the measurements were not normally distributed (Kolmogorov–Smirnov *D* Test).

As with the determination of the number of larval instars, a frequency distribution method was utilized to investigate and compare the measurements of PSHB and TSHB females. Histograms of pooled PSHB and TSHB measurements were plotted and kernel density estimation was used to determine the number of peaks, with each peak potentially representing a species.

Determination of cuticular hydrocarbon profiles of polyphagous shot hole borer and tea shot hole borer adults

PSHB were collected from naturally infested boxelder, *A. negundo californicum*, at a site near Whittier Narrows Nature Park, South El Monte, Los Angeles County, California, USA (N 34.03281°, W 118.07036°; March/April 2014 by TWC). This was the same general area where the species was discovered in California in 2003. Insects

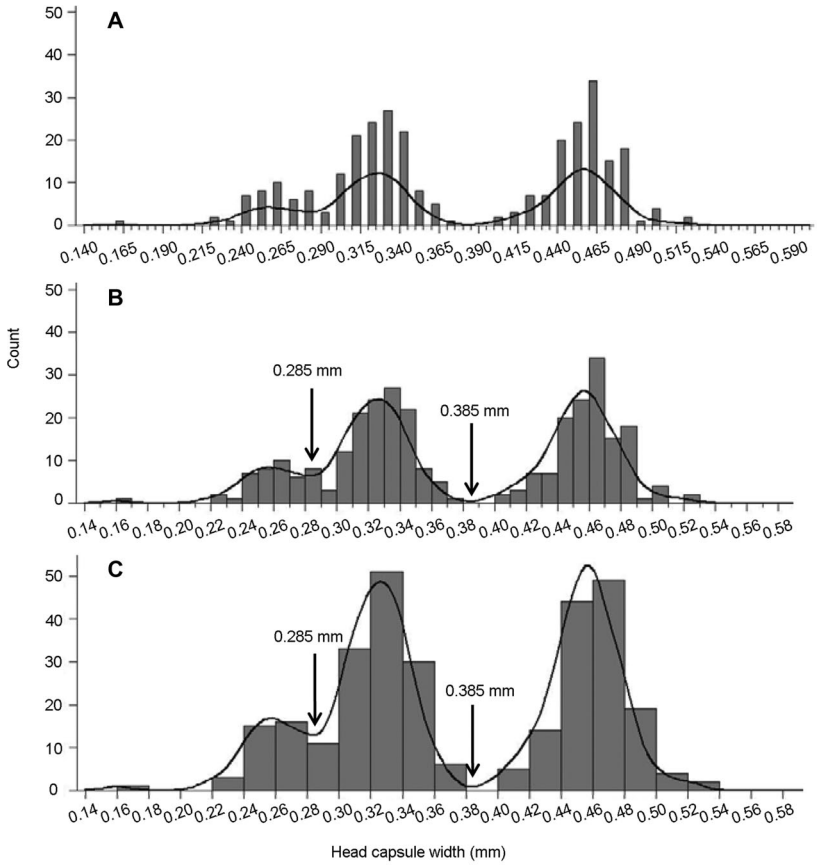

Fig. 2 Frequency distributions of larval *Euwallacea* sp. head capsule width and kernel density estimation at different width classes: (A) 0.005 mm, (B) 0.01 mm, and (C) 0.02 mm. Arrows show points of separation amongst peaks; $N = 303$.

Table 2 Parameter (i.e., a_i, b_i, and c_i) estimates of larval head capsule frequency distributions of the polyphagous shot hole borer, *Euwallacea* sp. (Coleoptera: Scolytidae).

Instar	Initial estimates			Initial NLLS			Final NLLS		
	a_i	b_i (mm^2)	c_i (mm)	a_i	b_i (mm^2)	c_i (mm)	a_i	b_i (mm^2)	c_i (mm)
1	18	1033.06	0.26	18.33	1283.70	0.26	18.57	1681.40	0.26
2	27	1730.10	0.33	27.13	1338.50	0.33	27.22	1417.30	0.33
3	34	1033.06	0.46	30.03	1484.90	0.46	30.03	1485.00	0.46

Note: The initial estimates for a_i, b_i, and c_i were derived from the counts of the most frequent width class, $1/(2{s_i}^2)$, and $\bar{x}_i$; the initial and final nonlinear least square (NLLS) estimates were derived from Equations (1) and (2), respectively, $N = 303$.

were stored at -35 ºC. Frozen insects were thawed, and immersed in 10 mL of hexane (EM Science, Omnisolv, Radnor, PA, USA) for 10 min to extract cuticular lipids. After extraction, hydrocarbons were separated from other compounds by pipetting the extract through 4 cm of activated silica gel (Sigma-Aldrich, St. Louis, MO, USA, 70–230 mesh) in Pasteur pipette mini-columns. An additional 5 mL of hexane was passed through the silica gel. The resulting hydrocarbon extracts were evaporated to dryness under a stream of nitrogen and redissolved in 60 μL of hexane for analysis by gas chromatography-mass spectrometry (GC-MS). A 3 μL aliquot was injected into the GS-MS.

GC-MS analyses were performed on an Agilent 6890 gas chromatograph coupled with the 5973 MSD, with Agilent Chemstation data analysis software G1701CA

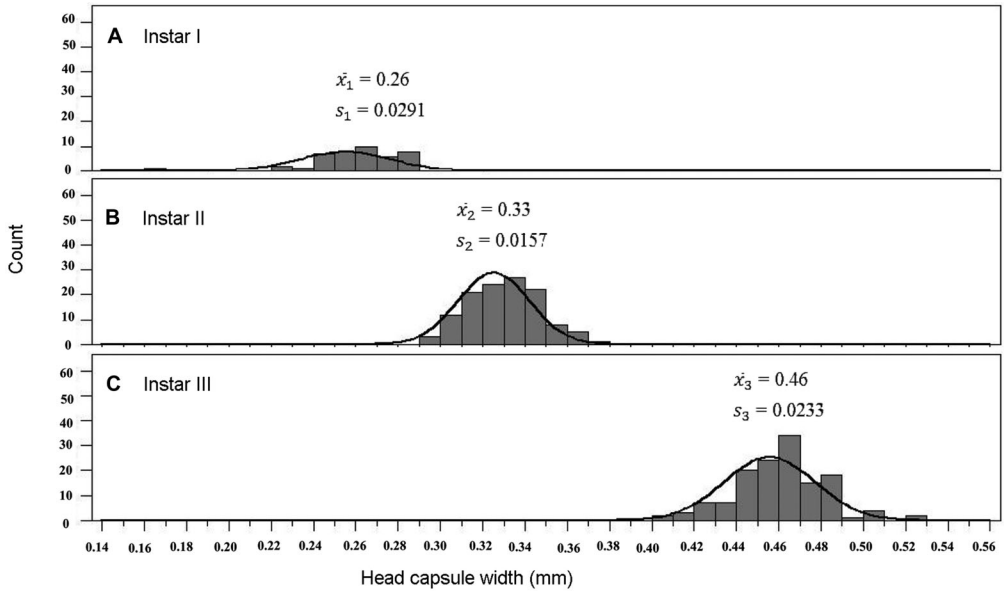

Fig. 3 Frequency distributions of larval *Euwallacea* sp. head capsules and their fitted normal curves. $\bar{x}_i$ and s_i were computed from their respective b_i and c_i that were obtained from the initial nonlinear least squares estimation (i.e., Equation [1]). (A) instar I, (B) instar II, and (C) instar III; $N = 303$.

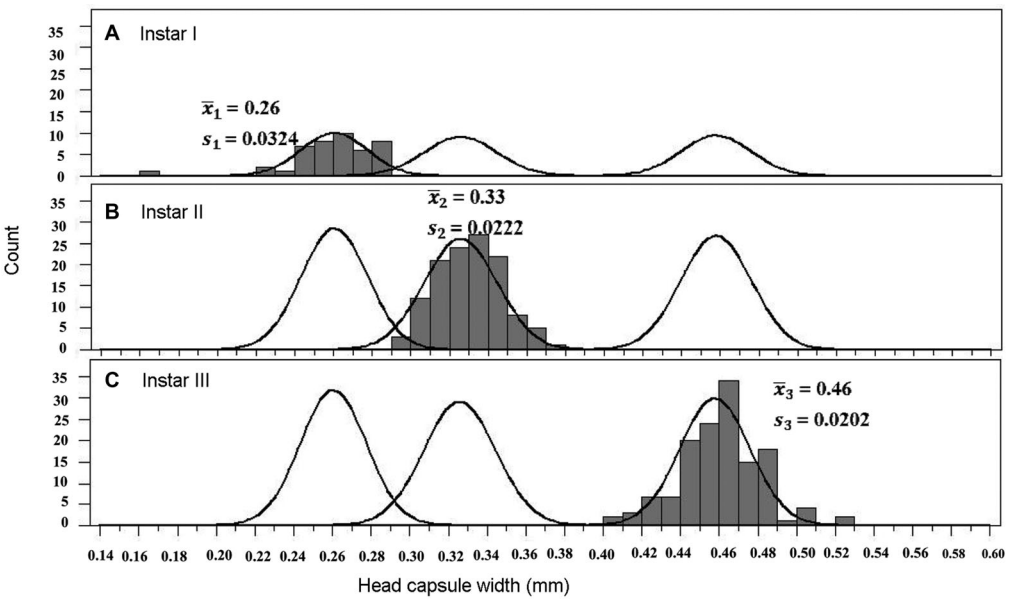

Fig. 4 Frequency distributions of larval *Euwallacea* sp. head capsules and their fitted normal curves. $\bar{x}_i$ and s_i were computed from their respective b_i and c_i that were obtained from the final nonlinear least squares estimation (i.e., Equation [2]). (A) instar I, (B) instar II, and (C) instar III; $N = 303$.

version D.01.02. The GC-MS was equipped with a nonpolar fused silica capillary column (HP-1MS; 30 m × 0.25 mm ID × 0.25 μm film thickness; Agilent Technologies, Wilmington, DE, USA) and operated in split mode (with a split ratio of 30 : 1). Helium was used as the carrier gas, at a flow rate of 1 mL/min. Each extract was analyzed by a temperature program from 200 ºC increasing to 320 ºC at 3ºC/min, with a final hold of 16 min. The injector temperature was 250 ºC. Electron impact (EI) mass spectra were obtained at 70 eV. *n*-Alkanes and

Table 3 Probabilities (%) of misclassifying polyphagous shot hole borer, *Euwallacea* sp. (Coleoptera: Scolytidae), larval instar *i* to *i* + 1 and larval instar *i* to *i* – 1 based on frequency distribution method.

Instar, *i*	Instar *i* to *i* + 1	Instar *i* to *i* – 1
1	16.17	–
2	0.00	4.35
3	–	0.00

Note: Probabilities calculated from Equations (4) and (5), $N = 303$.

Table 4 Means and standard deviations (SDs) of larval head capsule widths of polyphagous shot hole borer, *Euwallacea* sp. (Coleoptera: Scolytidae), and Brooks–Dyar growth ratios based on the theoretical frequency distribution.

Instar, *i*	Mean	SD	Growth ratio
1	0.26	0.0324	1.27
2	0.33	0.0222	1.39
3	0.46	0.0202	

Note: Brooks–Dyar growth ratios: ratio of consecutive instar head capsule widths.

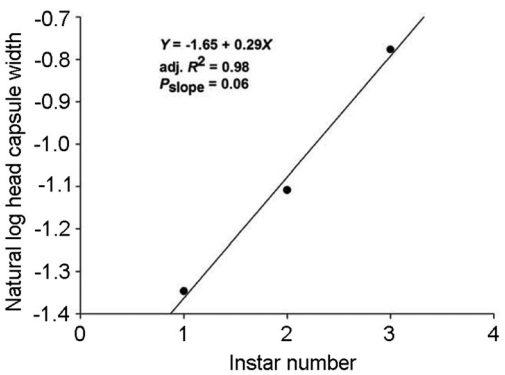

Fig. 5 Regression of the natural log of *Euwallacea* sp. head capsule width for each instar against the corresponding instar number.

methyl-branched alkanes were identified by their mass spectral fragmentation patterns (Blomquist *et al.*, 1987; Page *et al.*, 1990a,b; Nelson, 1993, 1997).

In the text and table, we use shorthand nomenclature to identify individual hydrocarbons or mixtures of hydrocarbons. This shorthand uses a descriptor for the total number of carbons (CXX) in the hydrocarbon component excluding the methyl branch(es), and the location of methyl groups (X-me). Thus, pentacosane becomes C25; 5-methylpentacosane becomes 5-meC25; and 5,17-dimethylheptacosane becomes 5,17-dimeC27.

Integration of the total ion chromatogram was performed by using Agilent Chemstation data analysis software. GC-MS peak areas were converted to percentages of the total hydrocarbon fraction. A summary table of the relative amounts of each peak for each species is presented, and the hydrocarbons are presented in order of elution under our conditions.

Cuticular hydrocarbon profiles were compared with a test of association (χ^2; PROC FREQ in SAS). We compared the cuticular hydrocarbon profile of 44 PSHB females collected in southern California with that of 43 TSHB females collected in Florida. We also tested whether there was sexual dimorphism in these characters in PSHB (145 males vs. 44 females) and whether the sample size of PSHB (44 females vs. 400 females) significantly affected the profile. We included more male than female PSHB in the comparative samples because males are smaller than females. Cuticular hydrocarbons were not compared between TSHB males and females due to lack of availability of males from the laboratory colony.

Voucher specimens of all adult material used in this study were deposited with the Department of Entomology, California Academy of Sciences, San Francisco, California, USA.

Results

Number of instars of polyphagous shot hole borer larvae

PSHB eggs and pupae were present in the galleries where PSHB larvae were collected. The head capsule widths of PSHB larvae ranged from 0.16 to 0.52 mm. Head capsule width classes of 0.01 (Fig. 2B) and 0.02 mm (Fig. 2C) fit the data better than did a class of 0.005 mm (Fig. 2A). A head capsule width class of 0.01 mm was used in the later analyses. Regardless of the head capsule width classes, kernel density estimation indicated 3 peaks (i.e., instars) (Fig. 2). The separation points for the 3 instars for the initial nonlinear least squares estimation were 0.285 and 0.385 mm (Fig. 2). Based on these separation points, the mean head capsule widths (± standard deviation) of the 3 instars were 0.26 (± 0.0291), 0.33 (± 0.0157), and 0.46 (± 0.0233), respectively (Table 2 and Fig. 3). These means (Fig. 3) from the initial nonlinear least squares estimation (Equation [1]) were the same as those in the final least squares estimation (Equation [2]) (Fig. 4), although the standard deviations were different (*cf.* Figs. 3 and 4).

The frequency distribution method separated distinctly the head capsule widths of the third instar PSHB from

Table 5 Cuticular hydrocarbons[†] extracted from 2 invasive *Euwallacea* spp.: polyphagous shot hole borer (PSHB) and tea shot hole borer (TSHB).

Peak No.	Compounds[‡,§]	PSHB[¶] (145 males)	PSHB[¶] (400 females)	PSHB[¶] (44 females)	TSHB[††] (43 females)
1	C27	0.15	0.23	0.00	0.00
2	11-meC27	0.00	0.00	0.00	0.46
3	14-meC28	0.00	0.00	0.00	2.27
4	C29	1.24	1.80	1.21	1.24
5	15-; 13-; 11-meC29	0.95	1.86	1.21	64.11
6	13,17-; 11,15-dimeC29	0.38	1.10	0.69	24.67
7	7,X-dimeC29+3-meC29	0.00	0.10	0.00	0.00
8	5,15-dimeC29	0.00	0.09	0.00	0.00
9	C30	0.00	0.30	0.00	0.00
10	15-; 14-; 13-; 12-meC30	0.00	0.34	0.00	0.95
11	13,17-; 12,16-dimeC30	0.42	1.09	0.61	0.38
12	C31	0.41	0.78	0.41	0.00
13	15-; 13-; 11-meC31	7.66	7.50	6.24	4.35
14	13,17-dimeC31; 7,19; 7,17-dimeC31	46.89	39.24	47.44	4.30
15	2-meC31; 5,15-; 5,17-; 5,19-dimeC31; 11,15,19-; 9,13,17-trimeC31	0.15	1.52	0.42	0.00
16	7,13,17-; 7,11,17-trimeC31	0.00	0.46	0.45	0.00
17	**5,X,X-trimeC31**	0.00	0.41	0.00	0.00
18	16-;15-;14-;13-;12-meC32	0.00	0.45	0.00	0.00
19	14,18-; 13,17-; 12,16-dimeC32	2.20	3.37	2.48	0.00
20	17-; 15-; 13-; 11-meC33	1.67	2.34	1.42	0.00
21	15,19-; 13,17; 11,21; **9,21**-dimeC33	35.59	31.41	34.23	0.00
22	5,15-; 5,17-; 5,19-; 5,21-dimeC33; 9,13-17-; 11,X,X-trimeC33	1.43	2.18	1.51	0.00
23	**5,X,X-trimeC33**	0.00	0.49	0.00	0.00
24	14,18-; 13,17-; 12,16-dimeC34	0.00	0.63	0.39	0.00
25	17-meC35	0.00	0.15	0.02	0.00
26	15,19-; 13,17-; 11,23-dimeC35	0.00	2.10	1.25	0.00
27	**11,17,23-trimeC33**	1.02	0.32	0.00	0.00

[†]Percentage of total hydrocarbon.

[‡]This shorthand uses a descriptor for the total number of carbons (CXX) in the hydrocarbon component, excluding the methyl branch(es), and the location of methyl groups (X-me) (see text).

[§]Cuticular hydrocarbons listed in bold type indicate tentative identifications.

[¶]*Euwallacea* sp. (PSHB) specimens were collected from logs collected in the spring (March/April) of 2014 near Whittier Narrows Nature Park (South El Monte) in Los Angeles County, CA. The developmental host was boxelder, *Acer negundo californicum.*

[††]*Euwallacea fornicatus* (TSHB) specimens were from a laboratory colony in Florida.

those of the first and second instars, and the probability of misclassifying the second instar as the third, or the reverse, was statistically 0 (Table 3). The distinction between the first and the second instars was less clear: the probabilities of misclassifying the first instar as the second instar and misclassifying the second instar as the first instar were 16.17% and 4.35%, respectively (Table 3 and Fig. 4).

The Brooks–Dyar growth ratio of the second instar to the first instar was 1.27 and that of the third instar to the second instar was 1.39 (Table 4). The natural log of the mean head capsule width of each instar and the corresponding instar number followed a linear relationship with an adjusted correlation coefficient of 0.98 (Fig. 5).

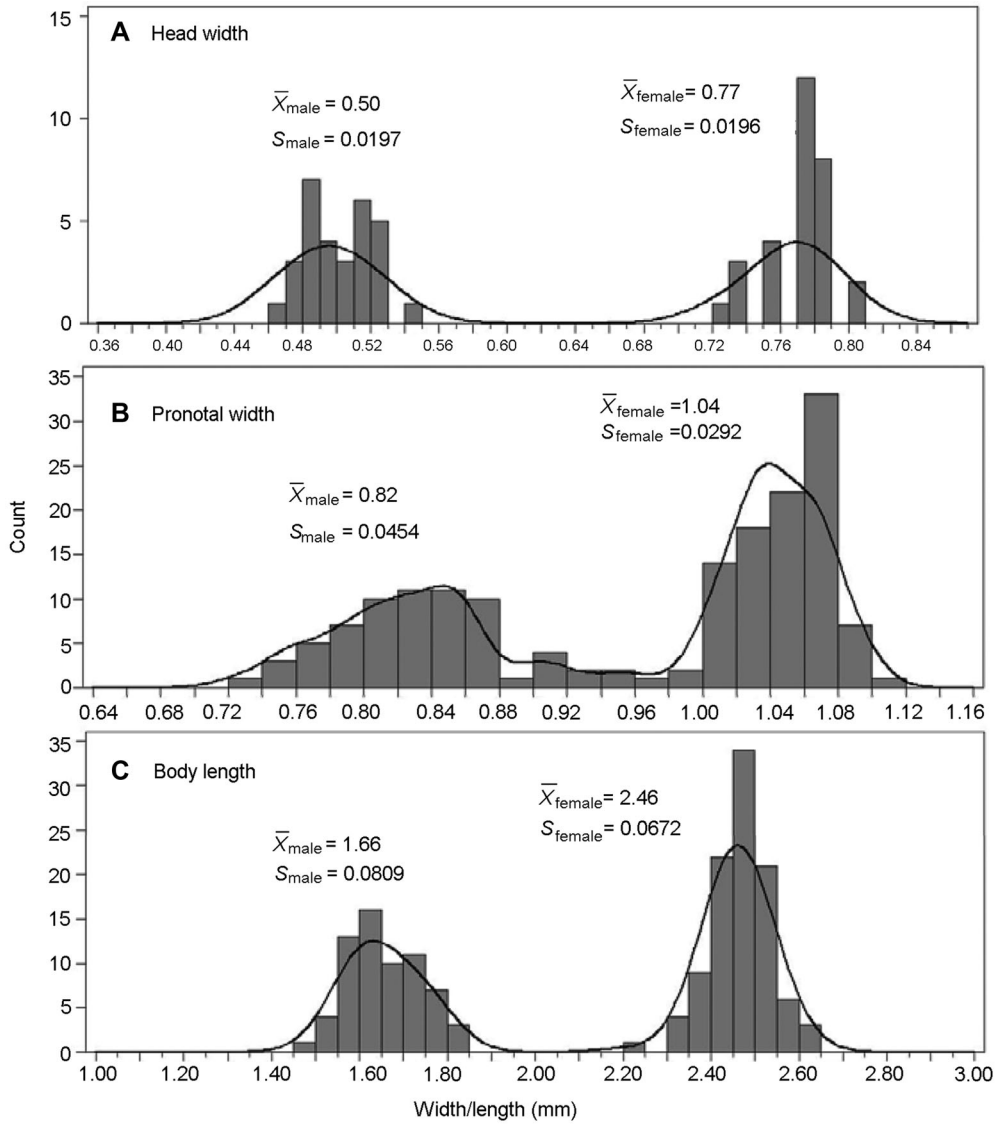

Fig. 6 Histograms and kernel density estimation of head width (A), pronotal width (B), and body length (C) of adult *Euwallacea* sp. $N_{male} = 35$ and $N_{female} = 35$ for head width; $N_{male} = 65$ and $N_{female} = 100$ for both pronotal width and body length.

Measurements of polyphagous shot hole borer and tea shot hole borer adults

The mean head width (± standard deviation) of PSHB males (0.50 ± 0.0197 mm, ranging from 0.46 to 0.54 mm), was significantly less than that of PSHB females (0.77 ± 0.0196 mm, ranging from 0.72 to 0.80 mm) ($\chi^2 = 44.90$, df = 1, $P < 0.001$). Kernel density estimation separated the head width data into 2 distinct peaks corresponding to each of the sexes (Fig. 6A).

The mean pronotal widths (± standard deviation) of PSHB males and females were 0.82 (± 0.0454) mm and 1.04 (± 0.0292) mm, respectively. The pronotal width of PSHB females (ranging from 0.95 to 1.10 mm) was significantly greater than that of PSHB males (ranging from 0.72 to 0.92 mm) ($\chi^2 = 118.28$, df = 1, $P < 0.001$). Although kernel density estimation suggested 2 peaks, the separation between sexes was not distinct (Fig. 6B).

The mean body lengths (± standard deviation) of PSHB males and females were 1.66 (± 0.0809) mm and 2.46 (± 0.0672) mm, respectively. The body length of PSHB females (ranging from 2.20 to 2.63 mm) was significantly greater than that of PSHB males (ranging from

1.50 to 1.82 mm) ($\chi^2 = 117.70$, df = 1, $P < 0.001$). Kernel density estimation separated the body length data into 2 distinct peaks corresponding to each of the sexes (Fig. 6C).

Head width of female PSHB (0.77 ± 0.0036 mm, ranging from 0.72 to 0.80 mm) was significantly less than that of female TSHB (0.80 ± 0.0014 mm, ranging from 0.78 to 0.81 mm) ($\chi^2 = 44.57$, df = 1, $P < 0.001$). Pronotal width of female PSHB (1.04 ± 0.0029 mm, ranging from 0.95 to 1.10 mm) was also significantly less than that of female TSHB (1.09 ± 0.0020 mm, ranging from 1.05 to 1.11 mm) ($\chi^2 = 64.02$, df = 1, $P < 0.001$). Body length of female PSHB (2.46 ± 0.0067 mm, ranging from 2.30 to 2.63 mm) was significantly greater than that of female TSHB (2.39 ± 0.0066 mm, ranging from 2.31 to 2.48 mm) ($t = 6.05$, df = 139, $P < 0.001$). The ratio of body length to pronotal width of PSHB females (2.26 ± 0.0056, ranging from 2.24 to 2.53) was significantly greater than that of TSHB females (2.20 ± 0.0059 mm, ranging from 2.12 to 2.30) ($\chi^2 = 84.80$, df = 1, $P < 0.001$). Although female PSHB and TSHB differed significantly in head width, pronotal width, body length, and ratio of body length to pronotal width, these 2 species could not be separated completely by these 4 measurements because of the overlapping ranges. Kernel density estimation did not produce distinct peaks for the 2 species (Fig. 7).

Cuticular hydrocarbon profiles of polyphagous shot hole borer and tea shot hole borer adults

Cuticular hydrocarbons detected in both species were exclusively alkanes (i.e., *n*-alkanes, monomethylalkanes, dimethylalkanes, and trimethylalkanes) (Table 5). Most of the peaks comprising methyl-branched alkanes were a mixture of coeluting isomers. The identifications were based on the mass spectral fragmentation patterns as described in Page *et al.* (1990a,b, 1997). Methyl-branched compounds with more internally located branch points (i.e., more symmetrical molecules) eluted earlier, and progressively scanning spectra across the width of the peak allowed distinction between the isomers. For example, in 13,17-dimeC31, fragments at *m/z* 196/197 and 224/225, where the even-to-odd ratio is greater than 1, arise from cleavage internal to each of the carbons bearing methyl branches. Predominant ion fragments at *m/z* 267 and 295 arise from cleavage external to the 2 branching methyl groups. At the trailing edge of this large peak, fragments at *m/z* 112/113 and 379 indicated the presence of a methyl branch on carbon 7. Normally under these GC conditions the 7, X-dimethylalkane would be resolved as a separate peak, however due to the great abundance of the 13,17 isomer these compounds co-eluted. In a few cases, insufficient quantity of a compound, or identical fragment ions for multiple isomers, precluded full characterization of methyl branching (Table 5).

Cuticular hydrocarbons of PSHB were dominated by peaks containing 13,17-; 7,19-; and 7,17-dimeC31 (peak #14), which ranged from 39.24% to 47.44% of the total hydrocarbon, and 15,19-; 13,17-; 11,21-; and/or 9,21-dimeC33 (peak #21), which ranged from 31.41% to 35.59% of the total (Table 5; Fig. 8). These peaks were 4.30% and 0.00% of the total hydrocarbon, respectively, in TSHB. The predominant peaks in the cuticular hydrocarbon profile of female TSHB were 15-; 13-; 11-meC29 (peak #5) at 64.11%, and 13,17-; 11,15-dimeC29 (peak #6) at 24.67% of the total (Table 5, Fig. 8). In PSHB, these peaks were each less than 2% of the total hydrocarbons.

The cuticular hydrocarbon profile of PSHB males was similar to that of PSHB females ($\chi^2_{17} = 3.78$, $P > 0.05$) (Figs. 8A and B). Cuticular hydrocarbons in the extract from 400 PSHB females contained trace amounts of 9 compounds that were not detected in the extract from 44 PSHB females. However, the cuticular hydrocarbon profiles in the extracts between the 400 and 44 PSHB females did not differ significantly ($\chi^2_{24} = 5.82$, $P > 0.05$) (Figs. 8B and C). The cuticular hydrocarbon profile in the extract of 44 PSHB females was significantly different from that of 43 TSHB females ($\chi^2_{18} = 165.86$, $P < 0.001$) (Figs. 8C and D). This is also evident from a visual examination of the total ion chromatograms (Figs. 8C and D).

Discussion

The number of instars of polyphagous shot hole borer larvae

Chen and Seybold (2013) suggested that the number of larval instars estimated by using the frequency distribution method may depend on the class width used to construct the frequency distribution histogram. Our analyses in the present study indicated that PSHB has 3 larval instars, irrespective of class width of the histogram (Figs. 2–4). The head capsule width measurement of 1 larva (0.16 mm) was far less than the rest. The reason for this outlier is unknown. The head capsule widths of the third instar were significantly greater than those of the first and second instars with no overlapping measurements (Figs. 2–4). The probability of misclassifying the third instar head capsules as the first 2 instars or vice versa was

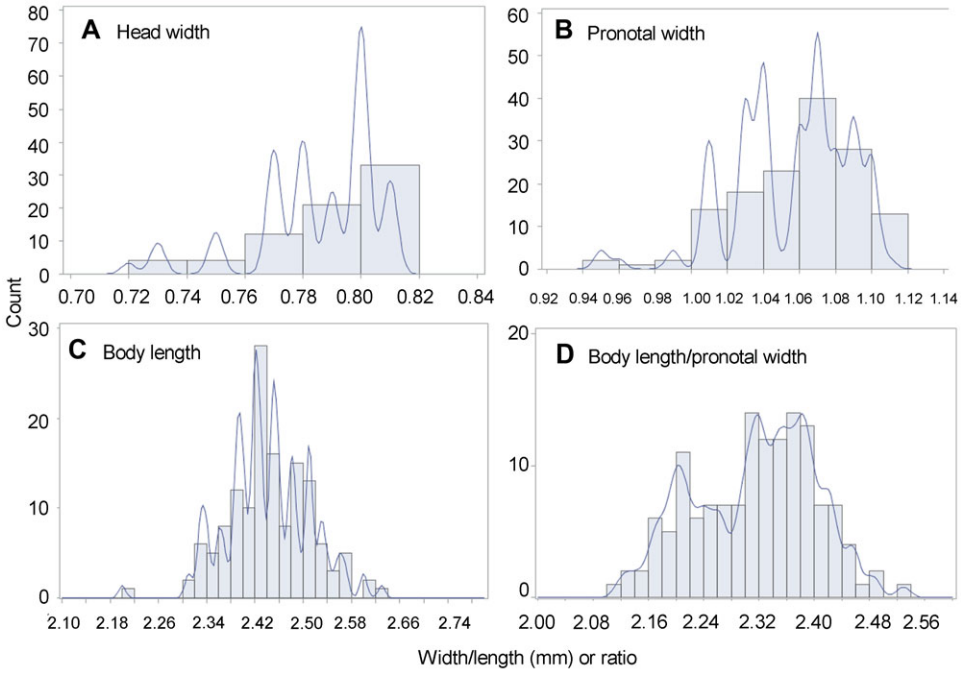

Fig. 7 Histograms and kernel density estimation of head width (A), pronotal width (B), body length (C), and ratio of body length to pronotal width of 2 adult *Euwallacea* spp. $N_{\text{head width}}$ =30 female polyphagous shot hole borers + 41 female tea shot hole borers; $N_{\text{pronotal width}}$ = 100 female polyphagous shot hole borers + 41 female tea shot hole borers; $N_{\text{body length}}$ = 100 female polyphagous shot hole borers + 41 female tea shot hole borers; $N_{\text{body length/pronotal width}}$ = 100 female polyphagous shot hole borers + 41 female tea shot hole borers.

0 (Table 2). The distinction between the first and second instar head capsules was less clear, and there is a possibility of misclassifying the first as the second instar or vice versa based on head capsule width (Table 2). The Brooks–Dyar rule growth ratios of the second instar to the first, and the third instar to the second instar were 1.27 and 1.39, respectively (Table 3). The proximity of these growth ratios is evidence that these 3 instars are 3 consecutive instars and that there were no instars missing in between. This was further confirmed by the linear relationship between the instar number and the natural log of head capsule width (Fig. 5). The presence of eggs and pupae in the galleries where the larvae were collected indicates that the first and last instars were accounted for in the larval sample (Daly, 1985).

We were not able to compare larval head capsule width between PSHB and TSHB because no TSHB larvae were harvested from the laboratory colony. Kumar *et al.* (2011) reported that an Indian population of TSHB had 3 instars, and provided mean length and breadth of each larval instar. However, the details of how their measurements were made and what constituted "length" and "breadth" were not evident from their publication. The mean breadths reported by Kumar *et al.* (2011) for each instar (0.37, 0.44, and 0.60 mm) are greater than the corresponding measurements of mean head capsule widths for larval PSHB in our study. If Kumar *et al.* (2011) did indeed measure larval head capsule widths for TSHB, they are either extremely different from those for PSHB or they will need to be repeated by using more well described methods.

Measurements of polyphagous shot hole borer and tea shot hole borer adults

PSHB females and males have a large number of gross morphological differences (Figs. 1A and B). We have found that they can also be distinguished by head width and body length, but not by pronotal width. The minimum head width of PSHB females (0.72 mm) was 0.18 mm greater than the maximum head width of PSHB males (0.54 mm) (Fig. 6A). The minimum body length of PSHB females (2.20 mm) was 0.38 mm greater than the maximum body length of PSHB males (1.82 mm) (Fig. 6C). The range of body lengths of females reported in this study (2.20–2.63 mm) is less than that reported elsewhere (1.80–2.50 mm; http://cisr.ucr.edu/polyphagous_shot_hole_borer.html; sample sizes

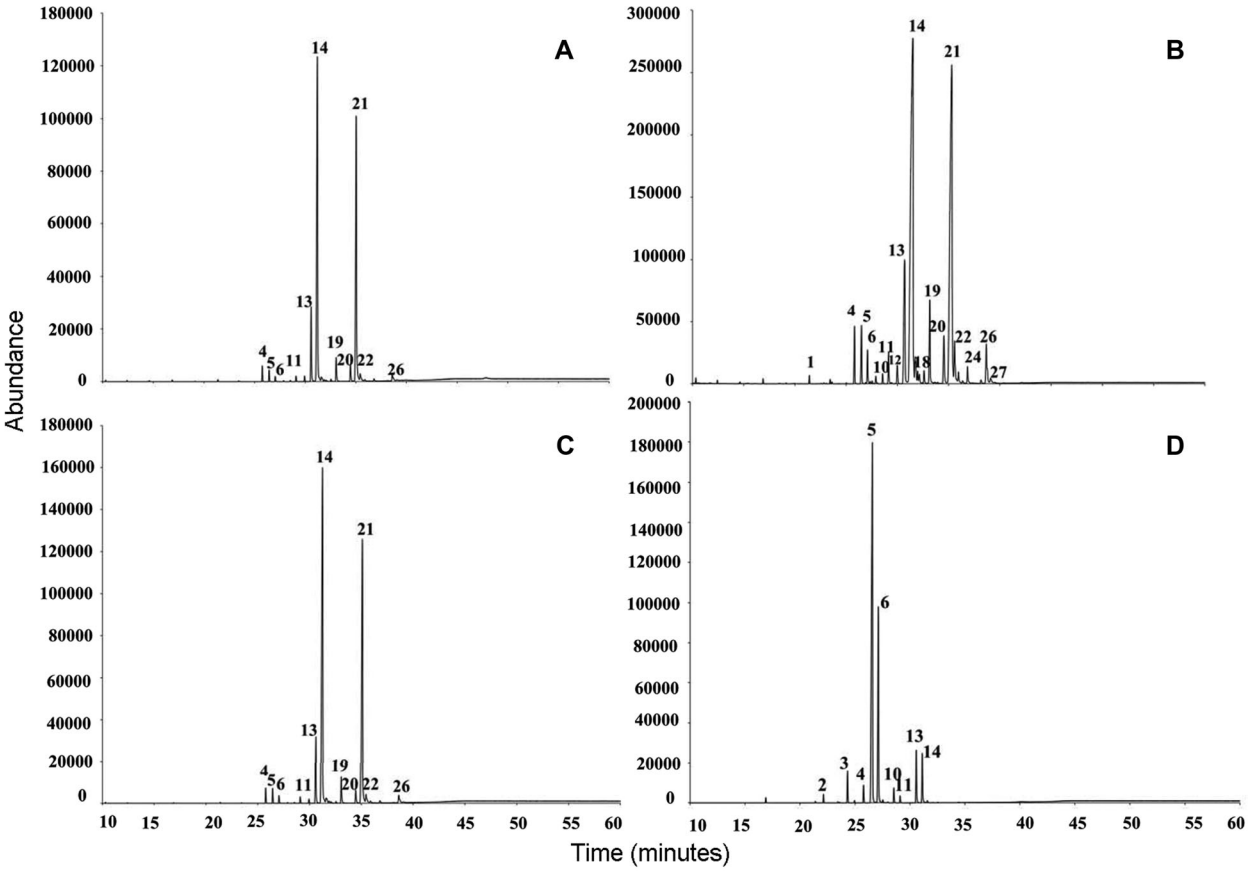

Fig. 8 Total ion chromatograms of cuticular hydrocarbons of *Euwallacea* sp. and *E. fornicatus*. See Table 5 for identification of the peaks. (A) 145 *Euwallacea* sp. males; (B) 400 *Euwallacea* sp. females; (C) 44 *Euwallacea* sp. females; and (D) 43 *E. fornicatus* females.

were unknown). The range of body lengths of males reported in this study (1.50–1.82 mm) is greater than that reported elsewhere (1.50–1.67 mm; http://cisr.ucr.edu/polyphagous_shot_hole_borer.html). Although pronotal widths of most PSHB males and females were distinctive, there is overlap in the distribution of pronotal widths of males at the higher end of the range and pronotal widths of females at the lower end of the range. This could be problematic for complete distinction between males and females based exclusively on pronotal width (Fig. 6B).

Although body length and ratio of body length to pronotal width of PSHB females were significantly greater than that of female TSHB, and pronotal width and head width of female PSHB were significantly less than that of female TSHB, females of these 2 species could not be separated by these 4 measurements (Fig. 7). Thus, although adult PSHB appears to be narrower and longer than adult TSHB, additional measurements, may be necessary to reveal any true morphological differences between these taxa.

Cuticular hydrocarbons of polyphagous shot hole borer and tea shot hole borer adults

With the hexane extracts of PSHB, the number of cuticular hydrocarbons detected increased with the biomass of PSHB: 14 compounds from 145 PSHB males (estimated as 1.63 mg), 16 compounds from 44 PSHB females (estimated as 2.23 mg), and 25 compounds from 400 PSHB females (estimated as 20.27 mg). Cuticular hydrocarbons of PSHB males were qualitatively and quantitatively very similar to those of PSHB females, indicating no sexual dimorphism in this suite of characters and little evidence to hypothesize proximal recognition of brothers and sisters by gustation or olfaction related to these compounds.

All of the major cuticular hydrocarbons of PSHB and TSHB are internally branched methyl- and dimethylalkanes. The dominant hydrocarbons were peaks #14 and #21 and peaks #5 and #6 in PSHB and TSHB, respectively. Peak #21, which was greater than 31% of the total hydrocarbon in PSHB, was completely absent in TSHB

(Table 5). The relative amounts of these monomethylalkanes or dimethylalkanes (primarily those with carbon chain lengths of C31 and C33 for PSHB and C29 for TSHB) are diagnostic for the 2 types of shot hole borers. Besides the quantitative differences in cuticular hydrocarbons between these 2 species, there are qualitative differences: 16 compounds were detected in 44 PSHB females, whereas only 9 were detected in 43 TSHB females. The quantitative and qualitative differences support the molecular genetic data that these two are separate species (Eskalen *et al.*, 2013).

Cuticular hydrocarbon composition is thought to be controlled genetically (Ferveur, 2005; Martin *et al.*, 2008). However, nongenetic factors such as temperature, geographic distances, and diet can also affect the cuticular hydrocarbon profile (Liang & Silverman, 2000; Buczkowski *et al.*, 2005; Martin *et al.*, 2008), but the variation is usually quantitative rather than qualitative (Kather & Martin, 2012). Although both PSHB and TSHB originate from southern Asia, PSHB is likely from Vietnam and southern China, whereas TSHB is likely from Sri Lanka (http://caforestpestcouncil.org/wp-content/uploads/2008/07/Polyphagous-Shot-Hole-Borer.pdf). The invaded geographic regions where the beetles were collected in this study were also different: PSHB from Los Angeles County, California and TSHB from Miami-Dade County, Florida, USA. The factors, whether genetic or nongenetic, which influence the differences between the cuticular hydrocarbon profiles of PSHB and TSHB remain to be understood, but this relatively simple chemical procedure will provide a means of separating small samples of the 2 species for future research on their invasion biology. The differences in steroisomeric forms of the methyl-branched cuticular hydrocarbons between PSHB and TSHB and their bioactivity remain to be understood, and these hypothetical differences may further distinguish these 2 species (Hughes *et al.*, 2015; Ruther, 2015).

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