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Morphometric Analysis of Larval Instar Stages of Reared Red Palm Weevil, Rhynchophorus Ferrugineus (Oliver, 1790), (Coleoptera:Curcolionidae)

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Abstract

This study was conducted to explore alternative morphometric characters that could be used to estimate the larval age of red palm weevil (RPW), *Rhynchophorus ferrugineus.* **A total of 14 characters explored in the study. Morphological characters were measured using Image J software and data were analyzed using One-way analysis of variance (ANOVA), principal components analysis (PCA), and discriminant function analysis (DFA). One-way ANOVA excluded characters that are not different among instars, which were distal head band (DB), basal head band (BB), distal tail width (DT), tail ratio (TR), and head band ratio (HBR). All other characters were found to be significant at $p < 0.05$. For PCA, cumulative variations of 88.7% from the first two principal components, evidenced by sharp decrease in slope of the scree plot, has resulted from high loadings in dorsal surface area (DSA), total surface area (TSA), ventral surface area (VSA), head capsule width (HW), body length (BL) ranging from 0.955 to 0.984. For DFA, only one function explained a canonical correlation of 0.986 with 94.9% variation, and Wilk's Lambda statistics of (0.007). The highest character loadings were BL, DSA, and HW, and could be useful characters in distinguishing instar ages of RPW larvae.**

Keywords: Morphometrics, *Rhynchophorus*, instar, weevils

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Rhynchophorus ferrugineus, commonly known as red palm weevil (RPW), is an important pest of many palm species including the coconut (*Cocos nucifera*) sago, date, and oil palms (Murphy & Briscoe, 1999). As a major economic pest, the majority of available publications on RPW focused on characterization, ecology, and pest-control (Faleiro et al., 2001; Gunawardena & Bandarage, 1995; Dembilo & Jacques, 2015; Alnujiban et al., 2015; Azmi et al., 2014; Azmi et al., 2017; Soroker et al., 2005; Soroker et al., 2013) while other studies emphasized on the rearing methods for RPW (Al-Ayedh, 2011; El-Shafie et al., 2013; Mahmoud et al., 2015; Sharaby & Al-Dhafar, 2013). The eggs of RPW are laid in injured palm trees, through the petiole wounds or in new leaves to hatch into voracious-feeding larvae (Ul-Haq et al., 2018). The instar age of RPW larvae are note-worthy because models could be made to trace their origins and other aspects of growth patterns. Insights from this knowledge can be explored for more efficient pest control and bio-control measures having optimal time for intervention using insecticides and entomopathogenic nematodes (Norzainih et al., 2015).

There is a challenge in identifying the instar age of RPW larvae caught in the wild. Instar age is usually the controlled variable in many studies. Morphometric analysis of larvae is difficult due to lack of landmarks in the larvae body. Head capsule diameter was previously used to determine instar age (El-Shafie et al., 2013). It was shown that there were eight instars before pupation, and that there was a linear correlation between larval instar age and head capsule diameter. These findings were supported by another study that also reported eight instars before pupation and a linear correlation between head capsule diameter and age (Norzainih et al., 2015).

To date, there are no other studies exploring the use of other morphometric measurements as a way to estimate larval instar age. It is therefore interesting to study if there are other morphometric measurements that can be used to estimate larval instar age of RPW. This study explored fourteen morphometric characters of red palm weevils. In particular, the study determined alternative morphometric characters that distinguished larval instar stages of RPWs from each other and discriminated RPW larvae from those of other species.

Materials and Methods

Insect collection

Red palm weevils (RPW) were collected from the fronds and trunks of infested coconut *(Cocos nucifera)* trees in Bago Oshiro, Mintal, Davao City. Using purposive sampling method, RPW samples were collected on January 2019 from area depicted in (Figure 1).

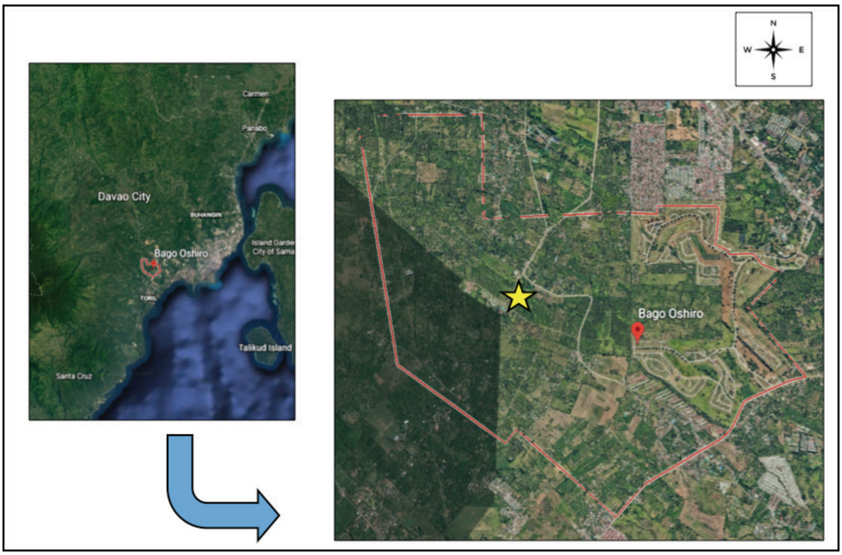

Figure 1. Map of Davao City, Philippines, showing the sample site in Bago Oshiro.

Farmers identified the infested coconut trees by loss of leaves and oozing amber brown liquid from the trunk consistent to symptoms as reported (Sharaby & Al-Dhafar, 2013). In addition, farmers also identified the presence of RPWs in the infested trees by hearing a dull sound upon knocking on the trunk. Infested trunks were cut to expose heart of palm where the RPWs congregated. These weevils were collected in sample bottles and were transported in the mass rearing laboratory of Philippine Coconut Authority in Bago Oshiro, Davao City. Adult specimens were identified, sorted, and introduced separately in labeled 340-mm x 200-mm x 125-mm rearing chambers containing coconut trunk with the heart of palm and covered by a mesh wire lid for aeration. The collection was indiscriminate between male and female RPW.

Species verification

RPW samples were verified by using guide by (Borror & White, 1970) and as reported by Abad, Ligad, and Aterrado (2008). Characters included distinct coloration of dorsal side of rostrum, clubbed and elbowed antennae, and absence of labrum. Although it was initially reported that *Rhynchophorus ferrugineus* (four-dotted dorsal rostrum) and *Rhynchophorus schachs* (red-striped dorsal rostrum) were different species, it was later elucidated using phylogenetic analysis and molecular profiling that the two are members of the same species exhibiting possible polymorphism (Abad et al., 2014).

Rearing

The rearing chambers were placed in cabinets of a low-light mass-rearing laboratory maintained at 25^{o}C and 70% relative humidity. Upon adulthood, male and female RPWs were transferred to another rearing chambers where they were left to mate and females to lay eggs in fresh coconut trunk. The eggs were allowed to hatch into larvae and pupate into adulthood. Subsequent generations of RPW larvae were grown in this manner. Routine replacement of coconut trunk was done every two weeks to replenish food supply and avoid fungal and bacterial growth. This was done with concurrent collection of larvae, pupa, and adults from the worn trunk. Collected individuals were reintroduced to another rearing chamber with fresh coconut trunk.

For this study, RPW larvae were already reared one generation from the original wild sample population. Photographs were taken during a routine replacement of coconut trunk. Available larvae were taken from rearing chambers, washed with distilled water, and then photographed individually at the dorsal and ventral sides. Photographs were taken alongside ruler for calibration of digital images.

Morphometrics

Photographs were processed using Image J software (Rasband, 2018). First, photograph was opened in Image J, then a calibration of known distance is set against a known number of pixels by setting global scale. Measurements of eight characters (BL, BW, SW, HW, BB, DB, DT, and MT) were then taken (Figure 2) by creating straight lines. Software analysis measured distance using global scale. Images were processed using magnetic lasso tool remove shadows to get the body surface area of the larvae (Figure 3) for DSA and VSA. The cropped body surfaces were then placed in a different layer with a differently colored background to highlight the images surface area measurement. This is done for both dorsal and ventral surfaces of the RPW larvae.

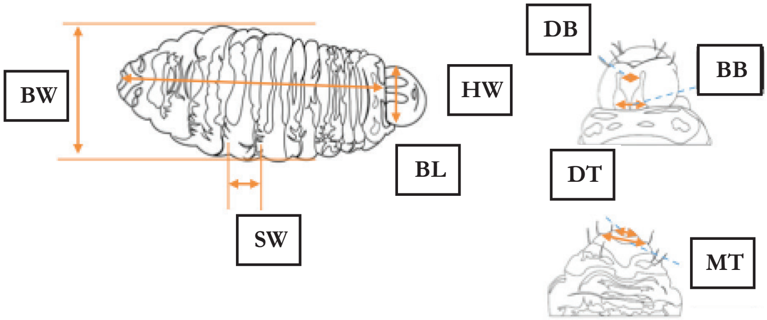

Figure 2. Measured morphological characters: full body diagram of RPW larvae in dorsal side, head (upper right) and tail (lower right) parts of RPW larvae.

Figure 3. Body surface (a) dorsal, (b) ventral of RPW larvae, cropped in computer software to respectively estimate DSA and VSA.

The following 14 morphological characters were measured: head capsule width (HW), Body width (BW), Body length (BL), Segment width (SW), Distance of distal head band (DB), Distance of basal head band (BB), Distal tail length (DT), Medio-distal tail length (MT), Dorsal surface area (DSA), and ventral surface area (VSA). Total surface area (TSA) is the total surface area of the body that includes the sum of both dorsal and ventral surface areas. Body ratio (BR) is the quotient of BL and BW. Tail ratio (TR) is the quotient of MT and DT. Head band ratio (HBR) is the quotient of DB and BB. Derived characters, such as ratio of two observable characters, were used in morphometric classifications (Calle et al., 2002).

Statistical Analysis

One-way ANOVA with Tukey-test was done using SPSS to all character to identify if there was significant difference between measurements at different instar ages. The box and whiskers plot was made using SPSS and was used to compare the ranges of each character, as well as the differences and overlapping between the ranges of characters per instar stage.

Principal component analysis (PCA) was done using SPSS to condense multivariate data into its main representative features by projection of the data into two-dimensional presentation. The analysis was based on the correlation matrix. For PCA, only the first two components were considered as per analysis of the scree plot.

Discriminant factor analysis was also done to define the significant characters for distinguishing instar age. The analysis was conducted following the procedures described by Sazali et al. (2018). Character loadings were performed all at once to investigate the integrity of pre-defined groups using the measure of distance of Wilk's Lambda.

Results

The study deduced 22 larvae in 8th instar, seven larvae in 7th instar, 10 larvae in 6th instar, two larvae in 5th instar, 11 larvae in 4th instar, and nine larvae in 3rd instar. In the work El-Shafie et al. (2013), RPW larvae were also reported to have eight instars before pupation. The first and second instars were not observed in the population, either because they were too minute to observe, or could not be harvested from rearing chamber without injuring them.

Descriptive data from 14 characters for RPW larvae are summarized in Table 1 including HW (head capsule width), BW (body width), BL (body length), SW (body segment width), DB (distance of distal head band), BB (distance of basal head band), DT (distal tail length), MT (medio-distal tail length) and SA (total surface area). Similar study on morphometric variations of bream (*Abramis brama orientalis*) used only six morphometric characteristics (Bani et al., 2015). Studies on adults of african palm beetles (*Rhynchophorus phoenicis*) considered seven characters (Tambe et al., 2013) and examined differences between *R. vulneratus* and *R. ferrugineus* using 23 morphological characters (Sazali et al., 2018).

Table 1. Descriptive statistics and significance scores from one-way ANOVA and Tukey test

Characters	N	Minimum (cm)	Maximum (cm)	Mean (cm)	Std. Deviation (cm)	Sig.
BL	61	.713	6.019	3.184	1.690	.000
BW	61	.187	2.789	1.134	.669	.000
DSA	58	.123	9.282	3.413	2.935	.000
VSA	58	.124	9.941	3.604	3.161	.000
TSA	61	.000	19.223	6.674	6.115	.000
DB	35	.000	.376	.199	.106	.110
BB	36	.000	.215	.086	.050	.182
SW	60	.000	1.000	.211	.294	.000
DT	51	.000	.288	.102	.106	.222
MT	51	.000	1.000	.293	.308	.002
BR	61	1.177	5.007	3.017	.604	.022
TR	51	.473	2.294	1.955	.262	.121
HBR	35	1.330	4.530	2.451	.597	.512
HW	61	.132	1.042	.519	.282	0.00

Table 1 also shows the results of the one-way ANOVA with Tukey test showing all variables are valid except in determining instar age, except DB, BB, DT, TR, and HBR. The characters were analyzed in boxplots, but were excluded from succeeding statistical analyses.

The present study also explored if boxplot diagrams can be used to compare the ranges of character measurements (Figure 4). Boxplot diagrams of characters that were mutually exclusive and had little to no overlap per instar were considered possible characters to determine instar age.

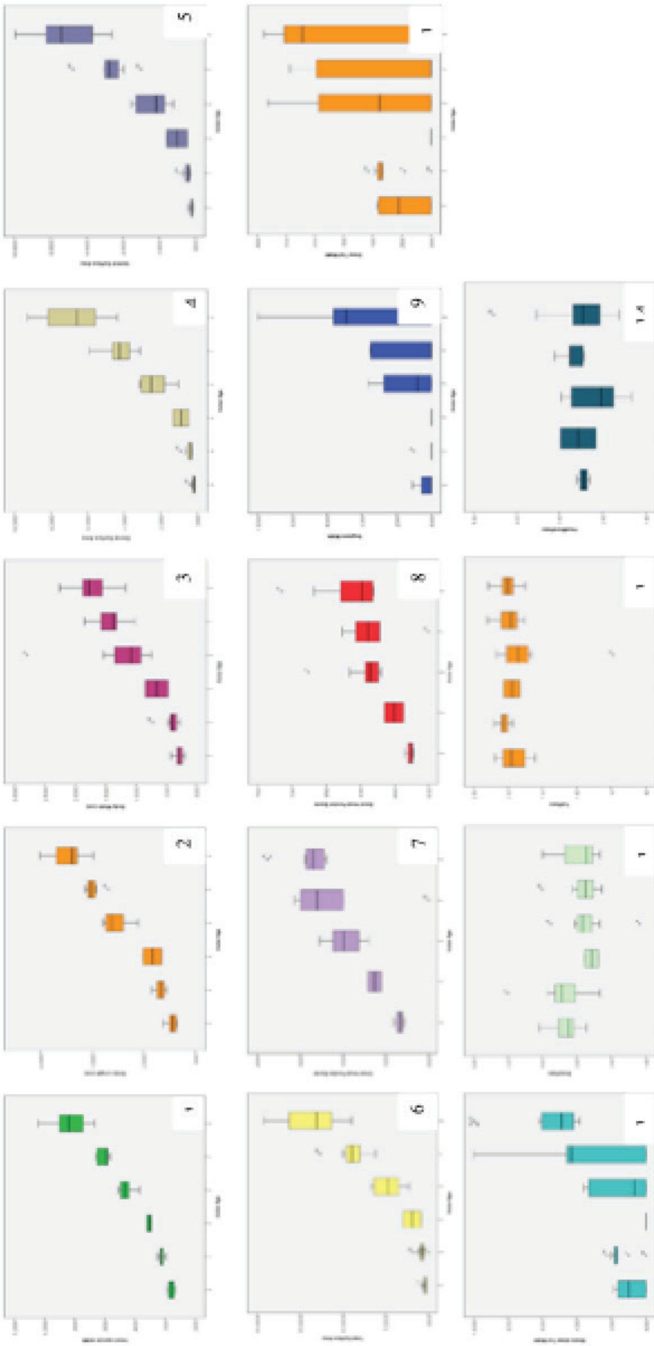

Figure 4. Boxplot diagrams of characters per instar age: 1 (HW), 2 (BL),5 (VSA), 6 (STA), 7 (DB), 9 (SW), 10 (DTI), 11(MT), 12 (BHBR)

In PCA, the first two principal components yielded the most variations among instars with cumulative variation of 88.7%. This is supported by scree plot from PCA (Figure 5). Only those with eigenvalues greater than 1 were considered.

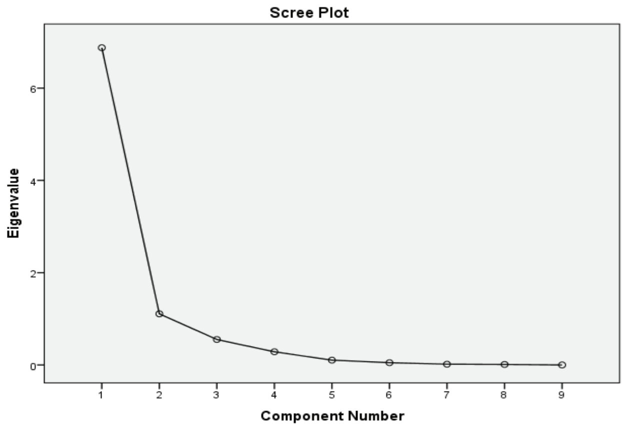

Figure 5. Scree plot from showing steep curve in first two factors

In the principal component 1 (Table 2) characteristics which showed higher loadings were DSA (0.984), TSA (0.984), and VSA (0.976) supported by eigenvalue of 6.875 and variation of 76.392%. On the other hand, principal component 2 had the following characters with the highest loading values BR (0.896), SW (0.404), and BL (0.081), supported by eigenvalue of 1.108 and variation of 88.698%.

Table 2. Characters and loading values from PCA

Character	Principal Component 1	Principal Component 2
BL	0.955	0.081
BW	0.908	-0.342
DSA	0.984	0.040
VSA	0.976	0.070
TSA	0.984	0.055
SW	0.769	0.404
MT	0.751	0.056
BR	-0.389	0.896
HW	0.970	0.069

In addition, DFA revealed one significant function that explains a canonical correlation of 0.986 which accounted for 94.9% of variance (Table 3). Further, the Wilks' Lambda score of 0.007 is supported by significance of $p<0.0001$ (Table 4). TSA was identified as character that failed tolerance test of level 0.001. Table 5 reveals that BL, DSA, and HW have high coefficient in Function 1.

Table 3. Eigenvalues for DFA

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	35.581	94.9	94.9	0.986

Table 4. Wilks' Lambda values for DFA

Test of function	Wilk's Lambda	Chi-Square	dF	Sig.
1 through 5	0.007	191.114	40	0.000

Table 5. Standardized canonical discriminant function coefficients for the eight characters

Character	Function 1
BL	1.007
BW	0.093
DSA	-0.976
VSA	0.105
SW	-0.270
MT	-0.269
BR	-0.295
HW	0.950

Discussion

In order to implement effective control of RPW, the knowledge on life history of this important pest such as instar aging is important. To date, there are no studies exploring the use of other morphometric measurements of RPW, aside from head capsule width (HW), as a means to estimate its larval instar age. HW has been reported as useful indicator of instar stage of larvae of moth, *Streblote panda*, but number of instar stages differed among host plants and varied from 5th to 8th instars (Calvo & Molina, 2009). It is also useful in determining larval stage of big avocado seed weevil, Heilipus lauri. Further, HW in Avocado seed weevil, *Heilipus lauri*, showed four distinct peaks after frequency analysis, and showed nearly no overlaps among the four instars in head capsule measurements (Castaneda-Vildozola et al., 2016).

Genetic variation is the variation in the DNA sequence in genomes that produce different phenotypes. On the other hand, Phenotypic plasticity is the epigenetic ability of one genotype to produce more than one phenotype when exposed to varying environments. The boxplots show some characters that appear to increase along with increase in instar age. This is similar to published works (El-Shafie et al., 2013; Norzainih et al., 2015). Meanwhile other characters do not exhibit this trend. Considering that RPW larvae were exposed to the same nutrition and environment, this could mean that differences in characters could be influenced by some factor. These differences in measurements could be explained by genetic variation. On the other hand, laboratory conditions could have induced smaller phenotypic expression among the larvae. But whether or not this factor is genetic variation, phenotypic plasticity, a combination of these two, or an entirely different phenomena, leaves to be explored.

Both principal component analysis (PCA) and discriminant factor analysis (DFA) found that the instar stages can be differentiated by body length (BL), dorsal surface area (DSA), and head capsule width (HW). While HW as character for instar age determination has already been published with good $R^2 = 0.978$ correlation (El-Shafie et al., 2013), BL and DSA are new possible characters to be considered in this application. This methodology has been used to identify characters that distinguish *R. vulneratus* from *R. ferrugineus* (Sazali et al., 2018).

However, a different study used ANOVA followed with Tukey test to differentiate polymorphism of male and female *R. phoenicis* (Tambe et al., 2013). On the other hand, phylogenetic analysis of DNA sequences was used to prove the synonymity of two previously different species of *R. ferrugineus* and *R. schach* (Abad et al. 2014).

Conclusion and Recommendation

Based on the findings of this study, it can be suggested that body length and dorsal surface area can be used to as characters to estimate the instar age of *R. ferrugineus* as an alternative to head capsule width. Although there is an increasing trend in characters with instar age, there seems to be an overlapping between measurements which shows an inconsistent pattern.

The study recommends expanding the sampling size and sampling areas to verify this finding. The study also recommends exploring factors leading to inconsistent and overlapping increase in character measurements. Furthermore, the study recommends exploring phylogenetic plasticity as an evolutionary mechanism among *R. ferrugineus* in determining larval duration and number of larval instars before pupation.

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