J. Entomol. Res. Soc., 20(3): 01-22, 2018 *Research Article*
Print ISSN:1302-0250 *Online ISSN:2651-3579*

Palm Weevil Diversity in Indonesia: Description of Phenotypic Variability in Asiatic Palm Weevil, *Rhynchophorus vulneratus* (Coleoptera: Curculionidae)

Sukirno SUKIRNO[1, 2*] Muhammad TUFAIL[1,3] Khawaja Ghulam RASOOL[1]
Abdulrahman Saad ALDAWOOD[1]

[1]Economic Entomology Research Unit, Plant Protection Department, College of Food and Agriculture Sciences, King Saud University, Riyadh 11451, KINGDOM OF SAUDI ARABIA. *Correspondence author's e-mail: sukirnobiougm@ugm.ac.id
[2]Entomology Laboratory, Faculty of Biology, Gadjah Mada University, Yogyakarta 55281, INDONESIA
[3]Organization of Advanced Science and Technology, Kobe University, Kobe 657-8501, JAPAN

ABSTRACT

Palm weevils (Coleoptera: Curculionidae) are the most destructive pest of many palm species worldwide, including Indonesia. Accurate species identification and knowledge of their diversity is crucial for implementing management strategies. Some samples of palm weevils from six main islands of Indonesia were found exhibiting intermediate color and markings between those of Asiatic palm weevil (APW), *Rhynchophorus vulneratus* Panzer and red palm weevil (RPW), *R. ferrugineus* (Olivier). To test the hypothesis that intermediate occurring phenotypes in Indonesia are only phenotypic color variations of APW, morphometric analyses, mating trials, and the analysis of cytochrome b (CyB) and random amplified polymorphism DNA (RAPD-PCR) were carried out. Additionally, RPW from the Kingdom of Saudi Arabia (KSA) and Pakistan were compared to Indonesian samples. Morphology-based identification of Indonesian palm weevils recognized three putative taxa: *R. ferrugineus*, *R. vulneratus,* and the black palm weevil, *R. bilineatus* (Montrouzier). It was suspected that intermediate color phenotypes of APW from Indonesia had been ambiguously considered RPW. Discriminant function analysis of morphometric measurements indicated that an intermediate weevil, which was previously determined to be RPW, are highly similar to APW. The mating trials of intermediate weevils and APW produced fertile progenies for three successive generations. The CyB and RAPD-PCR analyses showed that the intermediate phenotypes previously identified as RPW are distinguishable from RPW from the KSA. Therefore, our findings indicate that these are color phenotypes of APW. Thus, based on the palm weevil samples in this study, only two species exist, namely, *R. vulneratus* and *R. bilineatus*.

Key words: Morphometric, palm weevils, Indonesia, phenotype, polymorphic.

Sukirno, S., Tufail, M., Rasool, K.G., Aldawood, A.S. (2018). Palm weevil diversity in Indonesia: Description of phenotypic variability in Asiatic Palm Weevil, *Rhynchophorus vulneratus* (Coleoptera: Curculionidae), *Journal of the Entomological Research Society,* 20(3), 01-22.

INTRODUCTION

Palm weevils are large curculionid beetles belonging to the subfamily Rhynchophorinae. This subfamily consists of seven species that infest a wide range of palms (Booth *et al*., 1990). Five species, including red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier); African palm weevil, *R. phoenicis* (Fabricius); black palm weevil, *R. bilineatus* (Montrouzier); palmetto weevil, *R. cruentatus* (Fabricius), and South American palm weevil, *R. palmarum* (L.) are reported to be the most damaging pests. Among these species, *R. ferrugineus* is considered to be the most invasive pest of palms worldwide (Soroker *et al*., 2005; Malumphy and Moran, 2007; Bozbuga and Hazir, 2008; EPPO, 2008; Pelikh, 2009; USDA-CPHST, 2010; CABI, 2015). Based on the last five years studies in South East Asia, the RPW distribution has been evaluated and was confirmed that it exists in Thailand, Philippines, Vietnam, Cambodia, and several states in Malaysia (Azmi *et al*., 2013; Rugman-Jones *et al*., 2013).

In Indonesia, palm weevils have been relatively poorly studied. This is most likely because the Indonesian Ministry of Agriculture (IMA) has declared it, based on surveys in East Java during 2007 and 2008, as secondary pests only (DITJENBUN, 2010). Recently, the consequences of palm weevil infestations have become evident, and a high degree of damage to coconut palms has been reported by several districts of East Java, i.e., Ponorogo, Kediri, Jombang, and Probolinggo (Ernawati and Yuniarti, 2013; Wibowo and Ernawati, 2013; Trisnadi, 2014). Beginning 2016, the weevil has been declared the second most damaging pest of coconut in East Java region (Nadiah, 2016; Santosa, 2016).

Identifications of palm weevils from Indonesia have been open to question. IMA has reported three palm weevils: red palm weevil, *R. ferrugineus*, which has black spots on the pronotum; Asiatic palm weevil (APW), *R. vulneratus* or *R. schach,* which is black with a red pronotal stripe; and black palm weevil (scientific name not provided by source), which lacks pronotal spots (DITJENBUN, 2010). Kalshoven (1981) and Pracaya (1991) recognized these weevils as *R. ferrugineus*, *R. vulneratus,* and *R. bilineatus*, respectively.

A previous study on the taxonomy of palm weevils confirmed that these three species were present in Indonesia (Wattanapongsiri, 1966). However, there has been confusion about the identification of *R. vulneratus* in Indonesia (Santosa, 2016) and neighboring countries. For example, in Malaysia, this taxon has sometimes been considered *R. ferrugineus*, or the South American palm weevil, *R. palmarum* (L.), or *R. schach* (Wattanapongsiri, 1966).

The wide range of colors and markings exhibited by palm weevil adults has been a challenge for researchers and taxonomists in making conclusive identifications of these beetles, including those from Indonesia (Hallett *et al*., 2004; Rugman-Jones *et al.,* 2013). Some specimens from Indonesia have been identified as *R. ferrugineus* based on morphology (Wattanapongsiri, 1966). After studies of aggregation pheromones and analysis by random amplified polymorphic DNA polymerase chain reaction (RAPD PCR) on these palm weevils from West Java, Indonesia, it was proposed that *R. vulneratus* was a synonym of *R. ferrugineus* because both were capable of mating and producing fertile progenies (Hallett *et al*., 2004; Hallet *et al.*, 1993). However, a study

by Rugman-Jones *et al.* (2013) using the mitochondrial cytochrome c oxidase subunit I gene (COI) and pronotal shape to analyze a larger number of samples of *R. vulneratus* from Indonesia (Java, Sumatra, and Bali) and *R. ferrugineus* samples from 18 countries, determined that *R. vulneratus* is a distinguishable species from *R. ferrugineus.*

The identifying characteristics of palm weevils having a rusty red body with a high variability of black pronotal markings, morphologically very similar to that of RPW, are inconclusive, like in the case of *R. palmarum* in Colombia (Lohr *et al.*, 2015). Although the previous studies of Rugman-Jones *et al*. (2013) have found a wide range of phenotypic chromatic variations in APW and RPW, they have not aided species descriptions or identification of their specimens. By analyzing APW samples, they concluded that genetically there was no evidence of RPW presence in Indonesia. Nevertheless, until now there was no proof that APW exhibits atypical phenotypic variation, that is, a rusty red body with black pronotal markings instead of the typical black body with a red pronotal stripe.

Unambiguous taxonomic identification of palm weevils, as well as knowledge of their diversity, is critical, as it is the foundation for appropriate pest management programs. Although efforts have been made to identify palm weevils, including those in Indonesia, there are unanswered questions. In the present study, sampling of palm weevil populations was carried out on the six main islands of Indonesia to provide a better understanding of palm weevil diversity and to evaluate the extent of color variation in *R. vulneratus* and the other closely related species, *R. bilineatus*. Samples were subjected to morphological measurements to identify the characteristics useful for weevil species discrimination. Additionally, genetic compatibility, CyB and RAPD-PCR analyses of these putative species were evaluated for morphometric confirmation. The approaches were designed to test if there are color phenotypes of APW from Indonesian samples that were perhaps ambiguously considered RPW.

MATERIALS AND METHODS

Palm weevil collection

Larval, pupal, and adult stages of palm weevils collected from seven provinces covering six main islands of Indonesia from June to September of 2012 and 2014 were used in this study (Table 1). From these, two hundred and thirty-seven individuals were collected from 25 localities (Fig. 1). The sampling was conducted by hand picking adults, pupa, and larva from infested coconut palms (*Cocos nucifera* L.), toddy (*Borassus flabellifer* L.), and/or sago (*Metroxylon sago* Rottb.). Palm weevil infestation was recognized by the typical symptoms of oozing sap and frass along the palm trunk, apical leaf dieback, and a collapsed crown (Abraham *et al*., 1998). Adults were kept in 96% ethanol in 50 ml conical tubes. Collected larvae were maintained on sugarcane, while pupae were kept in plastic boxes until emergence. The emerged adults were then preserved in 96% ethanol. Adults of RPW collected by using pheromone traps from date palm farms in Al ‘Ammariyah, Riyadh Region, the Kingdom of Saudi Arabia (KSA) and Khudai, Punjab Province of Pakistan, were used for species comparisons.

All of the representative specimens used in this study were deposited in the King Saud Museum of Arthropods (KSMA), King Saud University, Riyadh, KSA.

Morphometric studies

The weevils collected at each locality were separated based on color and black pronotal marking pattern, and were subsequently designated into three main color phenotypes as used by IMA (DITJENBUN, 2010) and Wattanapongsiri (1966): rusty red with various black spots on the pronotum (RRPW), black with a red stripe on the pronotum (APW), and black with or without a longitudinal stripe (BPW). The diversity of pronotal color patterns of all collected weevils was observed and the pattern types were documented. Relative abundance (RA) and percentage of incidence (I) of the color morphs were measured to determine the phenotypes diversity (Mizzi *et al*., 2009; Tambe *et al*., 2013).

We suspected that RRPW was a color phenotype of APW. RPW collected from the KSA and BPW collected from Indonesia were used as controls to determine the effectiveness of the morphometric species separation. Forty-nine morphometric characters were measured using a Dino-Lite Edge Digital Microscope, AM4815ZT (AnMo Electronics Corp., USA). The proportion of black spots existing on the pronotum was measured using Digimizer image analysis software, version 4.3.1 (MedCalc Software, Belgium). The morphological species identification was conducted using a determination key (Wattanapongsiri, 1966).

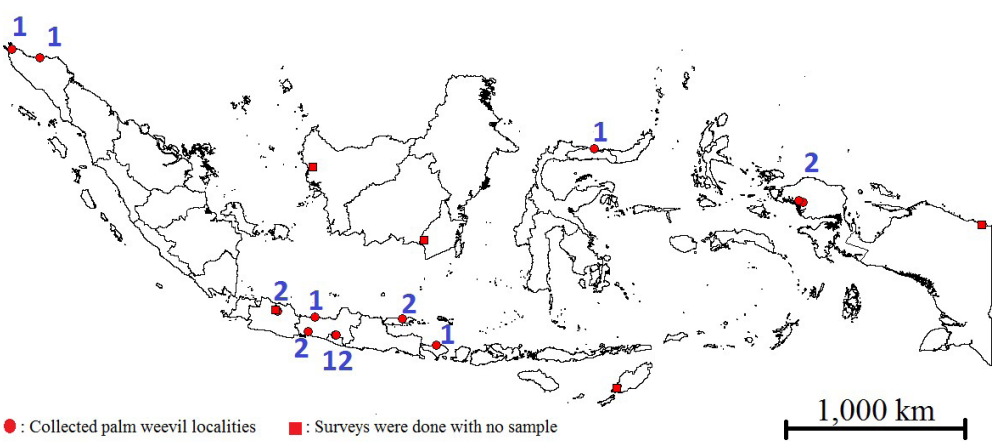

Fig. 1. Survey and collection sites of palm weevils from Indonesia, showing the number of localities at each site (● : localities where palm weevils were found/collected, ■ : localities where surveys were made but weevils were not found).

RRPW and APW mating experiment

Twenty-six palm weevil pupae were collected from coconut palms in Kebonalas, Klaten, Central Java, and brought to the laboratory. The pupae were kept individually in a plastic jar (d: 80 mm, h: 80 mm) covered with a perforated lid, and incubated at room temperature. The mating experiments were performed as heterospecific pairings

(RRPW x APW, RRPW x RRPW, and APW x APW) using unmated males and females. These mating experiments were repeated three times, whereas conspecific mating was performed twice.

Table 1. Localities (latitude and longitude) of palm weevil collections covering seven provinces in Indonesia, and several locations in Saudi Arabia and Pakistan.

No	Locality/Province*)	Code	Latitude	Longitude	Species
1.	Bireun, Aceh IN	BIR A	5.195158	96.71058	*R. vulneratus*
2.	Lambrita, Aceh IN	LAM A	5.576197	95.38698	*R. vulneratus*
3.	Katiasa, Bali IN	BAL A	-8.16072	115.1387	*R. bilineatus*
4.	Bumi Ayu, Central Java IN	BAY A	-7.53806	109.1794	*R. vulneratus*
5.	Jetis A, Central Java IN	JTS A	-7.66841	110.4987	*R. vulneratus*
6.	Jetis B, Central Java IN	JTS B	-7.66782	110.4967	*R. vulneratus*
7.	Jetis C, Central Java IN	JTS C	-7.66781	110.4965	*R. vulneratus*
8.	Jetis D, Central Java IN	JTS D	-7.66857	110.4988	*R. vulneratus*
9.	Jetis E, Central Java IN	JTS E	-7.66852	110.4973	*R. vulneratus*
10.	Kebon Alas A, Central Java IN Java	KBN A	-7.68646	110.4885	*R. vulneratus*
11.	Kebon Alas B, Central Java IN	KBN B	-7.68688	110.4885	*R. vulneratus*
12.	Kebon Alas C, Central Java IN	KBN C	-7.68708	110.4884	*R. vulneratus*
13.	Gondang, Central Java IN	GDG A	-7.67528	110.4967	*R. vulneratus*
14.	Pemalang, Central Java IN	PEM A	-6.85977	109.5075	*R. vulneratus*
15.	Madura A, East Java IN	MAD A	-6.94456	113.5491	*R. vulneratus*
16.	Madura B, East Java IN	MAD B	-6.93524	113.5553	*R. vulneratus*
17.	Sumalata, Gorontalo, Sulawesi IN	SUM A	0.974586	122.5041	*R. vulneratus*
18.	Aitinyo, West Papua IN	AIT A	-1.46081	132.0229	*R. bilineatus*
19.	Moswaren, West Papua IN	MOS A	-1.50742	132.233	*R. bilineatus*
20.	Teminabuan, West Papua IN	PAP A	-1.44905	132.0236	*R. bilineatus*
21.	Pendowoharjo, Jogjakarta IN	PND A	-7.68413	110.4341	*R. vulneratus*
22.	Kuwung A, Jogjakarta IN	KUW A	-7.67367	110.4581	*R. vulneratus*
23.	Kuwung B, Jogjakarta IN	KUW B	-7.67388	110.4581	*R. vulneratus*
24.	Alamariyah A, Riyadh SA	AMR A	24.80447	46.50868	*R. ferrugineus*
25.	Alamariyah B, Riyadh SA	AMR B	24.81748	46.51401	*R. ferrugineus*
26.	Khudai A, Punjab PK	KUD PK1	30.059377	71.179379	*R. ferrugineus*
27.	Khudai B, Punjab PK	KUD PK2	30.059377	71.179379	*R. ferrugineus*
28.	Khudai C, Punjab PK	KUD PK3	30.059377	71.179379	*R. ferrugineus*
29.	Muzafargarh, Punjab PK	MZF PK1	30.059377	71.179379	*R. ferrugineus*

Note: *) IN: Samples from Indonesia, PK: Samples from Pakistan, SA: Samples from Saudi Arabia.

RRPW and APW mating experiment

Twenty-six palm weevil pupae were collected from coconut palms in Kebonalas, Klaten, Central Java, and brought to the laboratory. The pupae were kept individually in a plastic jar (d: 80 mm, h: 80 mm) covered with a perforated lid, and incubated at room temperature. The mating experiments were performed as heterospecific pairings (RRPW x APW, RRPW x RRPW, and APW x APW) using unmated males and females. These mating experiments were repeated three times, whereas conspecific mating was performed twice.

Each pair was kept separately in a one liter plastic box (l: 160 mm, w: 100 mm, and h: 70 mm) covered with a perforated cover. Cotton saturated in a 10% sugar solution was provided as a food source. This cotton also served as a substrate for egg laying and allowed the weevils to remain upright. The observation and collection of eggs laid were carried out daily. The collected eggs from each pairing were kept in a plastic cup (d: 50 mm, h: 70 mm) and provided with water-saturated filter paper. The eggs were incubated at room temperature and their hatchability was observed daily. The hatched larvae were collected daily, then transferred to sugarcane for feeding and rearing (Kaakeh *et al*., 2001; Shahina *et al*., 2009). The interbreeding follow-up was conducted for one month, beginning on the first day of egg-laying by each pair. The experiment was repeated with three successive generations using these interbred progeny. Information on larval duration, pupal duration, full-grown larval weight, and adult weight of *R. vulneratus* on sugarcane was noted.

Identification using CyB and RAPD-PCR markers

Thirty-one specimens for the representatives of *R. vulneratus* (RRPW, APW, and IPW), *R. ferrugineus* (RPW) from Saudi Arabia and Pakistan, and *R. bilineatus* (BPW) were used to confirm the morphological identifications using CyB and RAPD markers. The proteinase-K lysis method (Collard *et al*., 2007) with several modifications was used for extracting the DNA. Fifty-five microliters of proteinase-K lysis buffer [20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 400 mM NaCl, 0.3% SDS and 100 µM proteinase-K] were added to each dried sample in a thin-wall 200 µL PCR tube, vortexed vigorously for 10 sec, and spun in a microcentrifuge (IKA® mini G IKA® – Werke GmbH and Co. KG, Germany). The sample was then incubated in an aluminum block bath (Cool Thermo Unit CTU-Neo, Taitec Corp., Japan) at 55°C for 3 h. After incubation, the sample was kept at ambient temperature for 10 min, briefly vortexed for 10 sec, and finally centrifuged at 10000 rpm for 1 min (Hermle Z216 MK, Germany) at 25°C to precipitate the debris. The crude DNA samples were used directly for the subsequent DNA amplifications. Each of 1 µL the supernatant was used as a PCR template to amplify the CyB gene and the random polymorphic DNA.

The CyB gene amplification was carried out in a 30 µL volume of KOD FX Neo polymerase kit solution (Toyobo Co., LTD., Japan) containing 0.2 µM each of MCB 398 and MCB 869 primers (Verma and Singh, 2002) synthesized by IDT DNA technologies (IDT DNA, Belgium), and 1 µL of crude DNA template. The CyB was amplified in a thermocycler (GeneAmp® PCR System 9700 Applied Biosystem, USA) with a heated

lid. The amplification conditions were as follows: initial denaturation at 95°C for 2 min; then 45 amplification cycles of 98°C for 10 sec, 54°C for 30 sec, and 68°C for 40 sec; and a final extension at 68°C for 5 min, followed by 4°C for an indefinite time.

The polymorphic DNA amplification was carried out in a 30 µL volume of KOD FX Neo polymerase kit (Toyobo Co., LTD., Japan) containing each of 0.2 µM of ten-mers random primers (Gadelhak and Enan, 2005) synthesized by IDT DNA technologies (IDT DNA, Belgium), and 1 µL of crude template. The DNA was amplified in a thermocycler (GeneAmp® PCR System 9700 Applied Biosystem, USA) with a heated lid. The amplification conditions were as follows: initial denaturation at 95°C for 2 min; then 45 amplification cycles of 98°C for 10 s, 36°C for 1 min, and 68°C for 2 min; and a final extension at 68°C for 5 min, followed by 4°C for an indefinite time.

For the analysis of amplicon, one microliter each of unpurified CyB amplicon was checked using 1% gel agarose electrophoresis at 100 V for 25 min in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) in an electrophoresis system (Mupid® - 2 Plus Submarine electrophoresis system Takara, Japan). The 1 kb Plus DNA Ladder (Invitrogen Life Technologies, USA) was used as a marker. The gels were then placed in an ethidium bromide solution (4 µL per 200 mL 1× TAE). Staining was performed by gentle shaking for 30 min at 45 rpm on an orbital shaker (Orbital incubator SI 500 Stuart®, Bibby scientific Ltd., UK). The stained gels were visualized under UV light in a gel documentation system (BioDocAnalyze, Biometra, Denmark). The amplified PCR products of CyB (~500 bp) were sent to Beijing Genomics Institute (BGI China) for sequencing in both directions using the Sanger method.

The RAPD-PCR amplicons were checked using 1.8% agarose gel, with the same electrophoresis, staining, and observation procedures as described above for the CyB analysis. The 1 kb Plus DNA Ladder (Invitrogen life technologies, USA) was used to estimate the molecular size of the amplicons. The presence of bright and reproducible bands was scored as one (1) while their absence was scored as zero (0). The score was only given to amplicons that had clear bands, and were subsequently converted to binary data by using the Jaccard index for the further construction of a phylogenetic tree (Sneath and Sokal, 1973). Finally, hierarchic clustering analysis using SPSS 13.0. was performed (SPSS Inc., 2005).

Both directions of the CyB sequences were aligned separately in BioEdit ver. 7.2.2 (Hall, 1999). The primers were trimmed to obtain 421 bp nucleotides. Basic local alignment search tool (BLAST) of the nucleotides was carried out using NCBI GenBank databases to confirm the obtained CyB sequences. The validated sequences were then aligned using ClustalW multiple alignments in the BioEdit program (Hall, 1999). The Kimura 2-parameter model available in MEGA 6.06 was used to estimate the pattern and rate of substitution, transition/transversion bias, and genetic diversity. This model was also used for constructing the neighbor-joining (NJ) phylogeny (Tamura *et al*., 2013) with 1000 bootstrap values. The transition and transversion substitutions were included in the analysis. Gaps or missing data were treated as complete deletions. A comparison of the NJ analysis based on the CyB and RAPD-PCR phylogenetic relationships from the same specimens was used for species identification.

Statistical analysis

A multivariate analysis of variance (MANOVA) at $\alpha = 0.05$ was conducted to determine whether there were significant differences between the three main phenotypes and RPW species based on the entire morphometric dataset. Subsequently, a one-way ANOVA was performed to evaluate the contribution of each characteristic in the color morph/species separations. Means were separate by least significant difference (LSD). Eleven morphometric characters contributing to strong color phenotypic differences were used for species separation. The morphometric and statistical analyses for species separation were based on (Sánchez-Ruiz and Sanmartín, 2000). All analysis procedures were performed using SPSS 13 (SPSSInc, 2005).

RESULTS

Palm weevil diversity in Indonesia

Based on morphological identification using a palm weevil identification key, three weevil species could be identified in Indonesia: RPW, *R. ferrugineus* (Fig. 2) APW, *R. vulneratus* (Fig. 3), and BPW, *R. bilineatus* (Fig. 4). RRPW and APW populations were sympatric in the Aceh Special Province, West Java, Jogjakarta Special Province, Central Java, East Java, North Sulawesi, and Madura. They were sympatric on the same host plant, from either coconut or toddy palms. BPW was found only in Bali and Sorong of West Papua. It was found on coconut palms and sago in Bali and West Papua, respectively. No palm weevil were found infesting oil palm plantations in the South and West Kalimantan provinces. Additionally, coconut plantations in East Nusa Tenggara were also surveyed, but no palm weevil or infested palm was found.

Phenotypic color variation of palm weevils in Indonesia was high. For RRPW, 20 distinguishable color phenotypes could be separated (Fig. 2). The average percentage contribution of black spot pronotal markings to the total pronotal area for the above three main weevil color phenotypes was 29%, 68%, and 99%, respectively (Table 2). Twelve RRPW color phenotypes were commonly found in both sexes ($M_{RR}1$-$M_{RR}12$) (Fig. 2), whereas eight color phenotypes were identified as male-specific ($M_{RR}13$-$M_{RR}20$). This suggests a higher tendency for color variation in males (20 color phenotypes) than in females (12 color phenotypes). In addition, $M_{RR}7$, with an average spot area of 19%, had the highest incidence (30%) in all localities, while the color phenotypes $M_{RR}11$, $M_{RR}12$, $M_{RR}13$, $M_{RR}14$, $M_{RR}15$, and $M_{RR}19$ represented only 4%.

In the case of APW, eight color phenotypes were recognized from Indonesia (Fig. 3). Five pronotal markings were common in both sexes ($M_{RS}1$-$M_{RS}5$) while the other three color phenotypes were found to be male-specific ($M_{RS}6$-$M_{RS}8$). Moreover, color morph 4 ($M_{RS}4$) presented the highest incidence (30%) in the 25 localities, while the color phenotypes $M_{RS}3$, $M_{RS}6$, and $M_{RS}8$ had very low incidence values (only 4%). In addition, $M_{RS}4$ was the most abundant color morph (25%) of the total collected individuals. Four individuals (3 females and 1 male) from North Sulawesi exhibited

a hybrid color pattern, suggesting the possibility of RRPW and APW hybridization. These weevils exhibited a rusty red color but with a red stripe on the pronotum ($M_{RS}3$).

BPW were collected from two localities (Bali and Sorong of West Papua) and four color phenotypes were observed (Fig. 4). Among these, two were commonly found in both sexes (M_B1-M_B2), whereas one was male-specific (M_B3) and one was female-specific (M_B4). We observed that the M_B1 color morph was found to be the most abundant in the collected samples (contributed 41%) while M_B4 (with two reddish longitudinal lines) was the least abundant (8.33%).

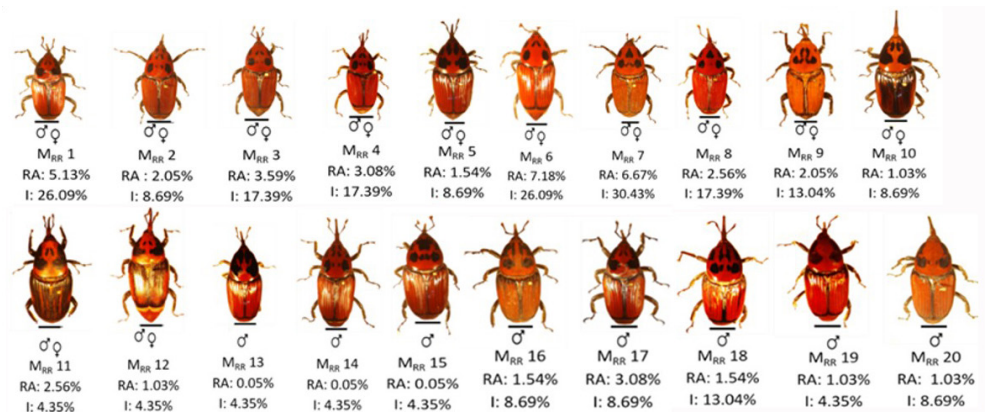

Fig. 2. Rusty red palm weevil (RRPW) color polymorphisms collected from Indonesia. Twenty rusty red color phenotypes ($M_{RR}1$-$M_{RR}20$) were collected/identified from Indonesia. $M_{RR}1$-$M_{RR}12$ are common in both males and females, while $M_{RR}13$-$M_{RR}20$ are male specific. RA and I indicate the percentage (%) of relative abundance and incidence, respectively (— bar: 5mm).

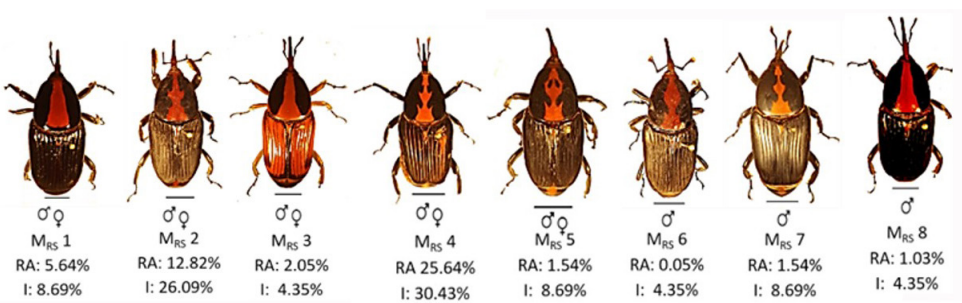

Fig. 3. Asiatic palm weevil (APW) polymorphisms collected from Indonesia. Eight red stripe color phenotypes ($M_{RS}1$-$M_{RS}8$) were collected/identified from Indonesia. $M_{RS}1$-$M_{RS}5$ are common in both males and females, while $M_{RS}6$-$M_{RS}8$ are male specific. $M_{RS}3$ are weevils representing a cocktail of characteristics, with a rusty red color and a red stripe. RA and I indicate the percentage (%) of relative abundance and incidence, respectively (— bar: 5mm).

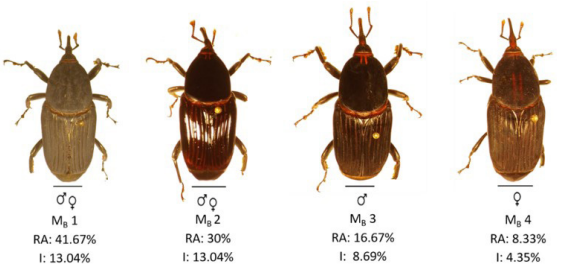

Fig. 4. Black palm weevil (BPW) polymorphisms collected from Indonesia. Four black color phenotypes (M_B1-M_B4) were collected/identified from Indonesia. M_B1and M_B2 are common in both males and females, while M_B3 and M_B4 are male and female specific, respectively. RA and I indicate the percentage (%) of relative abundance and incidence, respectively (— bar: 5mm).

Morphometric analysis

The three palm weevils from Indonesia identified in the present study as RRPW, APW, and BPW were compared with RPW from the KSA, and were further analyzed based on their morphometric characters (Table 2). The table indicates the 49 morphometric characteristics with means and standard errors. All characteristics showed significant differences, except for onychium width, which was not significant (F=1.02, df=73, *P*=0.427). Our morphometric analyses indicated that 11 characteristics could be potentially useful for identification of these four palm weevil species: scutellum length (F=8.99, df=71, *P*<0.001), pronotum width at apex (F=14.43, df=79, *P*<0.001), profemur length (F=13.92, df=79, *P*<0.001), protibial width (F=19.33, df=79, *P*<0.001), 3rd protarsomere length (F=19.40, df=77, *P*<0.001), 3rd midtarsomere length (F=14.28, df=76, *P*<0.001), 1st protarsomere width (F=14.46, df=77, *P*<0.001), 2nd protarsomere width (F=17.25, df=76, *P*<0.001), 3rd protarsomere width (F=10.3, df=74, *P*<0.001), pygidium length (F=18.87, df=77, *P*<0.001), and the size of the black spots (F=133.7, df=33, *P*<0.001).

Although 48 morphometric measurements indicated significant differences among RRPW, APW, and BPW as compared to RPW from the KSA, the discriminant function analysis based on the 11 main characteristics (Fig. 5) placed RRPW and APW as overlapping clusters, whereas both of BPW and RPW were clearly separate clusters. Figure 5 showed that RRPW has higher morphometric similarities to APW than to RPW, and they are even further separated from BPW.

RRPW and APW color phenotypes interbreeding

The interbreeding data for three successive generations of the RRPW and APW color phenotypes is provided in Table 3. RRPW and APW were capable of mating and producing fertile progenies. The average period of newly hatched to pre-pupa, and pupae to adult was 55.2 days (n=22) and 34.8 days (n=22), respectively. The average weight of full-grown larvae, adult males, and adult females was found to be 6.5 g (n=27), 1.3 g (n=11), and 1.5 g (n=12), respectively.

Table 2. The means and standard errors of the morphometric measurements (in mm) of red stripe palm weevil (RRPW), Asiatic palm weevil (APW), and black palm weevil (BPW) collected from Indonesia and red palm weevil (*R. ferrugineus*) from KSA.

No.	Characters	RSPW[a]	N	RRPW[a]	N	BPW[a]	N	RPW[a]	N
1	Rostrum width on pterygia (WRP)	1.58 (0.02)c	28	1.25 (0.03b	44	1.16 (0.04)b	10	0.97 (0.04)a	40
2	Rostrum width between scrobes (WRS)	1.02 (0.02)a	28	1.68 (0.05)b	44	1.81 (0.04)b	10	1.61 (0.04)b	40
3	Rostrum length (LR)	9.18 (0.24)a	28	11.28 (0.28)bc	44	11.98(0.48)c	10	1.19 0.22)ab	40
4	Frons width between eyes (WF)	0.86 (0.02)a	28	0.96 (0.02)b	43	0.96 (0.03)b	10	1.13 (0.02)c	40
5	Scape length*) (LSC)	3.63 (0.07)a	28	4.09 (0.12)b	36	4.49 (0.19)b	10	3.61 (0.09)a	40
6	Scape width at maximum (WSC)	0.59 (0.01)a	28	0.70 (0.02)a	36	0.73 (0.02)a	10	0.59 (0.02)a	40
7	1st funicular joint length (LF1)	2.54 (0.12)a	26	2.99 (0.09)b	36	3.14 (0.16)b	10	2.60 (0.07)a	40
8	2nd funicular joint length (LF2)	0.72 (0.01)a	26	0.89 (0.03)b	36	0.86 (0.03)b	10	0.72 (0.03)a	40
9	Antennal club length (LC)	1.26 (0.03)a	25	1.42 (0.04)b	36	1.55 (0.07)b	10	1.19 (0.05)a	40
10	Antennal club width (WC)	1.84 (0.06)a	25	2.10 (0.06)b	36	2.10 (0.08)b	10	1.71 (0.05)a	40
11	Pronotum length at midline (LP)	12.23 (0.20)a	28	13.85 (0.35)b	44	14.26 (0.60)b	10	12.08 (0.19)a	40
12	Pronotum width at maximum (WP)	10.05 (0.25)a	28	11.75 (0.29)b	44	12.08 (0.53)b	10	9.98 (0.18)a	40
13	Pronotum width at base (WPB)	9.85 (0.23)a	28	11.49 (0.29)b	44	11.82 (0.51)b	10	9.28 (0.19)a	40
14	Pronotum width at apex*) (WPA)	3.99 (0.11)a	28	4.63 (0.10)b	44	5.16 (0.19)c	10	4.28 (0.07)ab	40
15	Scutellum length at midline (LS)	3.72 (0.10)a	28	4.63 (0.14)b	44	4.58 (0.19)b	10	3.88 (0.09)a	40
16	Scutellum width at base (WS)	2.07 (0.07)a	28	2.70 (0.09)b	44	2.85 (0.15)b	10	2.14 (0.04)a	40
17	Elytra width at maximum (WE)	6.51 (0.14)a	28	7.28 (0.16)b	44	7.19 (0.29)b	10	5.99 (0.15)a	40
18	Elytra length at maximum (LE)	14.58 (0.27)a	28	17.59 (0.42)b	43	17.95 (0.68)b	10	15.28 (0.20)a	40
19	Profemur length*) (LPF)	6.20 (0.12b)a	28	7.11 (0.19)b	44	8.28 (0.42)c	10	5.74 (0.12)a	40
20	Profemur width at maximum (WPF)	2.35 (0.06)a	28	2.65 (0.07)b	44	2.64 (0.11)b	10	2.31 (0.05)a	40
21	Protibia length (LPT)	6.11 (0.15)a	28	7.07 (0.19)b	44	7.70 (0.36)b	10	6.01 (0.15)a	40
22	Mid tibia length (LMT)	4.72 (0.12)a	28	5.82 (0.16)b	44	6.37 (0.30)b	10	4.52 (0.10)a	40
23	Hind tibia length (LHT)	5.70 (0.13)a	27	7.05 (0.19)b	44	7.69 (0.32)b	9	5.32 (0.13)a	40
24	Protibial width at maximum*) (WPT)	1.04 (0.03)a	28	1.29 (0.03)b	44	1.44 (0.07)c	10	1.27 (0.05)b	40
25	Mid tibia width at maximum (WMT)	1.07 (0.03)a	28	1.22 (0.03)b	44	1.24 (0.03)b	10	1.16 (0.04)ab	40
26	Hind tibia width at maximum (HMT)	1.09 (0.05)a	28	1.38 (0.05)b	44	1.36 (0.05)b	9	1.18 (0.02)ab	40
27	Protarsus length (PLT)	4.94 (0.12)a	28	5.79 (0.18)b	41	6.26 (0.24)b	10	4.89 (0.07)a	40
28	Mid tarsus length (MLT)	4.60 (0.11)a	23	5.51 (0.15)b	42	5.92 (0.26)b	10	4.37 (0.31)a	38
29	Hind tarsus length (HLT)	4.80 (0.11)a	24	5.53 (0.14)b	39	5.82 (0.26)b	9	4.74 (0.06)a	39
30	1st protarsomere length (PLT1)	1.06 (0.03)a	28	1.26 (0.06)ab	42	1.42 (0.07)b	10	1.07 (0.04)a	40

Table 2. Continued.

No.	Characters	RSPW[a]	N	RRPW[a]	N	BPW[a]	N	RPW[a]	N
31	2nd protarsomere length (PLT2)	0.65 (0.02)a	28	0.79 (0.02)b	42	0.81 (0.04)b	10	0.69 (0.02)a	40
32	3rd protarsomere length*) (PLT3)	0.96 (0.02)a	28	1.18 (0.04)b	42	1.37 (0.06)c	10	1.04 (0.04)a	40
33	1st mid tarsomere length (MLT1)	0.97 (0.03)a	26	1.19 (0.04)b	44	1.23 (0.07)b	10	1.03 (0.05)a	39
34	2nd mid tarsomere length (MLT2)	0.61 (0.01)a	25	0.75 (0.02)b	44	0.80 (0.04)b	10	0.62 (0.03)a	39
35	3rd mid tarsomere length *) (MLT3)	0.93 (0.02)a	25	1.12 (0.03)b	44	1.25 (0.06)c	10	0.98 (0.03)a	39
36	1st hind tarsomere length (HP1)	0.94 (0.03)a	27	1.11 (0.03)b	44	1.12 (0.06)b	9	1.04 (0.04)ab	40
37	2nd hind tarsomere length (HP2)	0.59 (0.01)a	26	0.70 (0.02)b	42	0.71 (0.03)b	9	0.62 (0.01)a	40
38	3rd hind tarsomere length (HP3)	0.89 (0.02)a	26	1.14 (0.07)b	42	1.24 (0.06)b	9	1.03 (0.05)ab	40
39	Pro pretarsus length (LT1)	2.46 (0.05)a	28	2.94 (0.10)b	41	3.23 (0.15)b	10	2.38 (0.07)a	40
40	Mid pretarsus length (LT2)	2.24 (0.11)a	24	2.91 (0.09)b	42	3.21 (0.13)b	10	2.46 (0.05)a	40
41	Hind pretarsus length (LT3)	2.56 (0.06)a	24	3.05 (0.10)b	38	3.36 (0.13)b	9	2.46 (0.06)a	40
42	Onychium length at mid line (LON)	0.91 (0.02)a	28	1.02 (0.03)a	44	1.16 (0.04)b	10	0.90 (0.05)a	40
43	1st pro tarsomere width*) (WT1)	0.89 (0.02)a	28	1.05 (0.03)b	42	1.21 (0.05)c	10	0.99 (0.05)ab	40
44	2nd pro tarsomere width *) (WT2)	0.86 (0.02)a	25	1.01 (0.03)b	44	1.16 (0.05)c	10	0.98 (0.03)ab	40
45	3rd pro tarsomere width *) (WT3)	0.84 (0.01)a	26	0.94 (0.02)b	42	1.04 (0.04)c	9	0.91 (0.03)ab	40
46	Onychium width (WON)	0.22 (0.02)a	28	0.23 (0.01)a	44	0.27 (0.02)a	10	0.23 (0.02)a	40
47	Body width at maximum *) (WL)	5.34 (0.24)a	27	6.93 (0.21)b	43	7.81 (0.29)c	10	6.85 (0.26)b	40
48	Pygidium length (TL)	12.48 (0.23)a	28	14.49 (0.34)b	44	14.54 (0.60)b	10	12.52 (0.28)a	40
49	Spots size*) (SPOTS)	68.64 (2.81)c	12	29.26 (2.15)b	20	99.74 (0.09)d	4	8.95 (3.55)a	20

*Numbers in the same row followed by the same letter showing no significant differences at α 0.05. The character no. 5, 14, 19, 24, 32, 35, 43, 44, 45, 47, and 49 indicate a strong separation for the three species. All the unit are in mm, except SPOTS that is in %. N indicates the number of measured individuals. a The numbers in paranthezis indicate the standard error of mean.

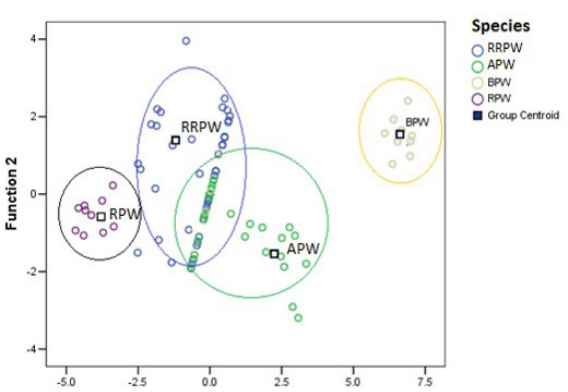

Fig. 5. The plots of first and second discriminant functions based on a set of 11 morphological measurements of rusty red palm weevil (RRPW), Asiatic palm weevil (APW), and black palm weevil (BPW) from Indonesia, and red palm weevil (*R. ferrugineus*) from Saudi Arabia.

Table 3. The means and standard errors of eggs produced and percentage of eggs hatching within one month of *R. vulneratus* color phenotypes mating for three successive generations.

Pairings	First Generation		Second Generation		Third Generation	
	Eggs laid[a]	Hatched (%)[a]	Eggs laid[a]	Hatched (%)[a]	Eggs laid[a]	Hatched (%)[a]
RRPW-APW (n= 3)	78 (10.1)	85 (8.2)	97.3 (10.6)	86.7 (7.2)	181 (26.9)	61.8 (7.85)
RRPW-RRPW (n= 2)	88.5 (56.6)	71 (16.3)	129 (65.7)	82.2 (26.3)	109 (20.5)	51.7 (8.3)
APW-APW (n= 2)	182 (28.3)	57.5 (7.9)	87.7 (28.9)	65.4 (11.9)	77.7 (10.6)	81.9 (4.9)

Note: RRPW-APW= cross breeding of rusty red palm weevil with red stripe palm weevil, RRPW-RRPW= in breeding of rusty red palm weevil, and APW-APW= inbreeding of red stripe palm weevil with red stripe palm weevil. n indicates the number of replicate. a The numbers in paranthezis indicate the standard error of mean.

Identification based on CyB

The primers amplified all the samples with a single unique band of ~450 bp. The primers were trimmed and clean sequences with a length of 421 bp were obtained. All of the CyB sequences have been deposited into the BOLD with the accession numbers PWINA133-16 to PWINA163-16 (accessible at http://boldsystems.org/). The genetic diversity analysis based on CyB showed that most of the weevil color morphs or species had identical sequences (d = 0), except for *R. ferrugineus* from Saudi Arabia, which had 0.001 genetic diversity (Table 4).

Table 4. Several species of *Rhynchophorus* collected from Indonesia, Saudi Arabia, and Pakistan used in this study, and their genetic diversity within the populations based on cytochrome b (CyB) gene analysis.

No.	Species (N)		N	Sample Code	Locality*)	CyB[a)]
1.	*R. bilineatus*	BPW	3	BAL–A1-3	Katiasa, Bali IN	0.000
2.	*R. vulneratus*	RRPW	2	KUW – C2; KUW– D1	Kuwung, Jogjakarta IN	0.000
3.	*R. vulneratus*	RRPW	6	MAD –A2-4; MAD –B1,3-4	Pamekasan, Madura IN	0.000
4.	*R. vulneratus*	IPW	2	SUM –A5-6	Sumalata, Gorontalo IN	0.000
5.	*R. vulneratus*	APW	5	KUW – E1-2,4-6	Kuwung, Jogjakarta IN	0.000
6.	*R. vulneratus*	APW	4	SUM –A1-4	Sumalata, Gorontalo IN	0.000
7.	*R. vulneratus*	APW	1	LAM –A1	Lambrita, Aceh IN	na
8.	*R. ferrugineus*	RPW	3	MZF – PK1-3	Muzafargarh, Pakistan PK	0.000
9.	*R. ferrugineus*	RPW	5	AMR –A3,4; MAD –B1-3	Alamariyah, Saudi Arabia SA	0.001

Note: N indicates the number of individuals used in the analysis. *) IN: Samples from Indonesia, PK: Samples from Pakistan, SA: Samples from Saudi Arabia. Numbers in column CyBA) indicate the genetic distance within the population, na: no available data because only had 1 sequence.

The study showed that based on the CyB marker, the interspecific variation was greater than intraspecific variation (Table 5). The genetic diversity of all *R. vulneratus* color morphs (RRPW, APW, and IPW) was zero. When compared to BPW (*R. bilineatus*) and *R. ferrugineus* from Saudi Arabia and Pakistan, the genetic distance was 0.241, 0.383, and 0.382, respectively. The genetic distance between

R. ferrugineus from Saudi Arabia and Pakistan was <0.001, while the distance was 0.130 when compared with BPW.

Table 5. Genetic distances between rusty red palm weevil (RRPW), Asiatic palm weevil (APW), intermediate palm weevil (IPW), and black palm weevil (BPW) phenotypes in Indonesia, and *Rhynchophorus ferrugineus* in Saudi Arabia and Pakistan, based on cytochrome b (CyB) gene.

Samples-Color Morphs	N	Locality*)	1	2	3	4	5	6	7	8
1.SUM-IPW	3	Sumalata, Gorontalo IN								
2. MAD-RRPW	2	Pamekasan, Madura IN	0.000							
3. KUW-RRPW	6	Kuwung, Jogjakarta IN	0.000	0.000						
4. KUW-APW	2	Kuwung, Jogjakarta IN	0.000	0.000	0.000					
5. SUM-APW	5	Sumalata, Gorontalo IN	0.000	0.000	0.000	0.000				
6. LAM-APW	4	Lambrita, Aceh IN	0.000	0.000	0.000	0.000	0.000			
7. BAL-BPW	1	Katiasa, Bali IN	0.241	0.241	0.241	0.241	0.240	0.240		
8. MZF-RPW	3	Muzafargarh, Pakistan PK	0.382	0.382	0.382	0.382	0.382	0.382	0.130	
9. AMR-RPW	5	Alamariyah, Saudi Arabia SA	0.383	0.383	0.383	0.383	0.383	0.383	0.130	0.000

Note: *) IN: Samples from Indonesia, PK: Samples from Pakistan, SA: Samples from Saudi Arabia. *R. vulneratus* color polymorphism representatives: 1 - intermediate palm weevil color morph; 2, 3 - rusty red palm weevil color morph; 4, 5, 6 - Asiatic palm weevil color morph. *Rhynchophorus bilineatus* only exhibited a completely black color morph (no. 7). *Rhynchophorus ferrugineus* color morph representatives: 10 - two-spotted RPW; 11 - three-spotted RPW; 3 - three-spotted RPW; 4 - seven-spotted RPW. N indicates the number of individuals used in the analysis. The genetic distances were calculated only using non-identical sequences from each population.

A neighbor joining analysis of CyB showed that the specimens strongly separated into species clusters. In this analysis, the specimens were separated into three clusters, designated as C1, C2, and C3, representing the three different species (Fig. 6). Cluster C1 contained all the *R. vulneratus* color morphs: RRPW, APW, and IPW. Meanwhile, clusters C2 and C3 comprised of *R. bilineatus* and *R. ferrugineus*, respectively.

Identification based on RAPD-PCR markers

The analysis of RAPD-PCR showed bands of 200-1900 bp. All amplified bands were polymorphic (Table 6). The genetic variation between the different color morphs and species was investigated using the Jaccard index distance (dJ) (Table 7). The highest genetic distance was found between RRPW from Madura, Indonesia, and two-spotted RPW from Khudai, Pakistan (dJ = 0.96). The lowest dJ index (dJ = 0.30) was found between two- and three-spotted RPWs from Khudai, Pakistan. The range of genetic distance between IPW and RRPW, RRPW and APW, RRPW and BPW, and APW and BPW was 0.5-0.75, 0.36-0.87, 0.60-0.84, and 0.66-0.75, respectively.

The UPGMA dendrogram showed four main clusters, which were designated as Ca, Cb, Cc, and Cd (Fig. 7). Most of the clusters correspond to the species (*R. ferrugineus, R. vulneratus,* and *R. bilineatus*). The cluster Ca was composed of *R. ferrugineus* samples from Pakistan and Saudi Arabia. The spotted RRPW, maple leaf RRPW, and APW color morphs were grouped into the cluster Cb. The RRPW

color morphs exhibiting bilaterally symmetrical shading and BPW (*R. bilineatus*) were separated into clusters Cc and Cd, respectively.

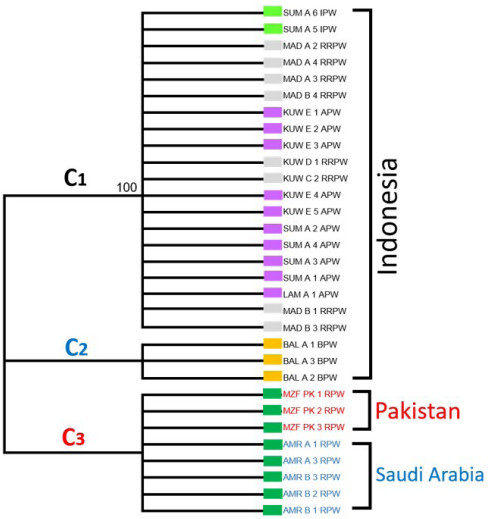

Fig. 6. The genealogical relationships of 31 sequences of the rusty red palm weevil (RRPW), Asiatic palm weevil (APW), intermediate palm weevil (IPW), and black palm weevil (BPW) from Indonesia and *Rhynchophorus ferrugineus* from Saudi Arabia and Pakistan, based on the 421 bp sequence of cytochrome b (CyB) gene using the neighbor-joining method with 1000 bootstraps. The numbers at the branching points indicate the bootstrap support values. The colors indicate the palm weevil phenotypes or species: ■ - rusty red palm weevil (RRPW), ■ - Asiatic or red stripe palm weevil (APW), ■ - intermediate palm weevil (IPW), ■ - black palm weevil (BPW), and ■ - red palm weevil (RPW). The bootstrap values below 50% were cut off.

Table 6. Total number of bands calculated from palm weevils representing *Rhynchophorus vulneratus* and *R. bilineatus* collected from Indonesia, and *R. ferrugineus* from Saudi Arabia and Pakistan.

Primers	Palm weevil species															
	R. vulneratus[a)]									*R. ferrugineus*[b)]					*R. bilineatus*	Total
	1	2	3	4	5	6	7	8		1	2	3	4			
Primer 1	7	7	4	2	7	7	3	6		1	2	3	3		4	62
Primer 2	1	1	4	4	0	4	2	3		2	2	2	2		4	33
Primer 3	2	2	1	2	0	1	1	1		4	4	4	4		1	27
Primer 4	0	0	4	4	4	4	1	5		3	4	2	2		3	36
Primer 5	0	1	0	0	1	1	1	1		1	4	0	5		0	15
Primer 6	1	0	3	1	5	1	4	2		3	3	0	3		4	30
Total	11	11	16	13	17	18	12	18		14	19	11	19		16	**203**

Note: The numbers in column a) indicate the *R. vulneratus* color morphs: 1, 2 - intermediate palm weevil morphs; 3, 4, 8 - rusty red palm weevil morphs; 5, 6, 7 - Asiatic palm weevil morphs. The numbers in column b) describe the R. ferrugineus morphs: 1 - two-spotted RPW; 2 - three-spotted RPW; 3 - three-spotted RPW; 4 - seven-spotted RPW. The R. bilineatus only used completely black-colored morph.

Table 7. Approximate genetic distances between rusty red palm weevil (RRPW), Asiatic palm weevil (APW), intermediate palm weevil (IPW), and black palm weevil (BPW) collected from Indonesia and *Rhynchophorus ferrugineus* collected from Saudi Arabia and Pakistan, based on the Jaccard index distance.

Specimen Code	1	2	3	4	5	6	7	8	9	10	11	12
1. SUMA5-IPW												
2. SUMA6-IPW	0.44											
3. MADA2-RRPW	0.50	0.50										
4. MADA4-RRPW	0.78	0.75	0.79									
5. SUMA1-APW	0.58	0.65	0.70	0.61								
6. SUMA4-APW	0.81	0.83	0.87	0.36	0.68							
7. KUW E1-APW	0.64	0.71	0.75	0.44	0.62	0.63						
8. MAD B4-RRPW	0.54	0.61	0.71	0.46	0.59	0.48	0.42					
9. BALA3-BPW	0.84	0.79	0.82	0.60	0.84	0.75	0.66	0.71				
10. RPW MZF PK1	0.84	0.87	0.96	0.58	0.70	0.80	0.70	0.76	0.81			
11. RPW MZF PK2	0.86	0.85	0.93	0.59	0.74	0.80	0.70	0.71	0.77	0.30		
12. RPW AMRA3	0.81	0.71	0.83	0.64	0.76	0.73	0.81	0.67	0.79	0.56	0.57	
13. RPW AMRA4	0.86	0.80	0.88	0.59	0.78	0.80	0.70	0.71	0.73	0.52	0.35	0.42

Note: *Rhynchophorus vulneratus* morph representatives: 1, 2 - intermediate palm weevil (IPW) morphs; 3, 4, 8 - rusty red palm weevil (RRPW) morphs; 5, 6, 7 - red stripe palm weevil morphs. The *R. bilineatus* specimens only exhibited a completely black-colored morph (no. 9). *Rhynchophorus ferrugineus* morph representatives: 10 – two-spotted RPW; 11 - three-spotted RPW; 3 - three-spotted RPW; 4 - seven-spotted RPW.

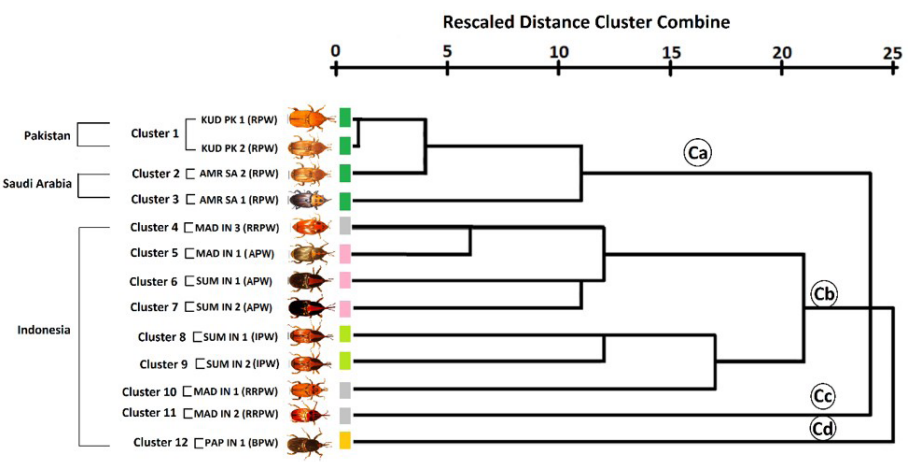

Fig. 7. Dendrogram showing the species separation of palm weevils collected from Indonesia and red palm weevils from Saudi Arabia and Pakistan, constructed based on the random amplified polymorphism DNA binary Jaccard index using UPGMA method. The colors indicate the following morphs: ■ - rusty red palm weevil (RRPW), ■ - Asiatic palm weevil (APW), ■ - intermediate palm weevil (IPW), ■ - black palm weevil (BPW), ■ - red palm weevil (RPW).

DISCUSSION AND CONCLUSIONS

Recently, severe infestations of palm weevils in coconut palms were reported in Indonesia (Ernawati and Yuniarti, 2013; Trisnadi, 2014; Ratmawati, 2015; Yuliyanto and Ernawati, 2015). It was believed that three species of palm weevils were present in Indonesia: *R. ferrugineus*, *R. schach* (a synonym of *R. vulneratus*), and *R. bilineatus* (Kalshoven, 1981; Pracaya, 1991; Wattanapongsiri, 1966). Due to the morphological observations that RPW and APW were only distinguishable based on pronotal shape and markings, Wattanapongsiri (1966) concluded there were indeed three species present in Indonesia.

However, we suspected that RRPW and IPW were the same species as APW. The results of morphometric characterization, mating experiments, CyB, and RAPD-PCR analyses support the findings that they were indeed color polymorphic of APW. These suggest that this study there were two species exist: *R. vulneratus* (covering RRPW and APW color phenotypes) and the black palm weevil, *R. bilineatus*. This is in agreement with Rugman-Jones *et al*. (2013) who conclusively proved that *R. ferrugineus* was not present in Indonesia. The ambiguity of species identity of spotted rusty red - palm weevils in Indonesia (Kalshoven, 1981; Pracaya, 1991; DITJENBUN, 2010; Hallett *et al*., 2004; Santosa, 2016), now has been solved by this study.

The phenomenon of color polymorphism has also been observed in black palm weevil (*R. palmarum*) from Colombia (Lohr *et al.*, 2015). It was found that the black palm weevil was completely black in color, except for nearly 0.3% of the captured weevils have a rusty red color closely similar to RPW. Their morphology and COI data confirmed that these specimens are color polymorphic of *R. palmarum*. The color variations may be a response to nutrition (Vanderplank, 1960), population density (Sword *et al.*, 2000), habitat conditions (Théry *et al.*, 2008), or substrate color (Hochkirch *et al.*, 2008).

The CyB marker has been widely used for species identifications. For examples, it has been used for Atlantic cod (*Gadus morhua*) (Carr and Marshall, 1991), Eurasian species of the genus *Mustela* (Mustelidae: Carnivora) (Kurose *et al*., 2000), several endangered animals in Taiwan (Hsieh *et al*., 2001), and the avian parasitic morphospecies *Haemoproteus* (Haemosporida: Haemoproteidae) (Barrientos *et al*., 2002). This marker has been proved as a sensitive and accurate tool for species identification, even with small amounts of degraded DNA material (Branicki *et al*., 2003).

Although the RAPD-PCR methods have limitation on the reproducibility (Penner *et al*., 1993; Micheli *et al.*, 1994; Skroch and Nienhuis, 1995; Jones *et al*., 1997; Bagley *et al*., 2001), the analysis suggested that it was a reliable method species identification, with the exception of the individual exhibiting the bilaterally symmetrical pronotum pattern. Beside its limitation, the method has some advantages over the other methods used in this study, as it does not require any prior knowledge on the particular gene in the targeted organism; furthermore, the use of more than one primer might also increase its efficacy (Calvert *et al*., 2005). It also has the added advantage of being cheaper because it does not require a subsequent sequence analysis.

Abad *et al*. (2014) studied a morphometric of *Rhynchophorus* from Philippines using the length and width of the head, thorax, abdomen, rostrum and antenna, femur, tibia, and tarsus. There were no significant differences reported by these authors. In this study, the multivariate discriminant analysis has shown as an effective method for distinguishing palm weevil species. The use of a single characteristic, or a small number of them, would not be sufficient for identification, especially for cryptic species. We propose that the overall eleven morphometric measurements should be analyzed as a whole for use in species identification.

Additionally, Chong *et al*. (2015) have conducted genetic diversity study of RPW in Terengganu Malaysia using COI marker. It showed that all samples had identical sequences and they suggested that it was originated from a single haplotype introduction from Mediterranean countries. In Malaysia, the RPW was firstly reported as a pest of coconut palm in east coast of the country (Azmi *et al*., 2013). The separation of APW and RPW as distinct species have been done using cytochrome oxidase sub unit I (COI) (Rugman-Jones et al., 2013; Yong, 2016), and distinguishing *R. ferrugineus* to *R. phoenicis, R. cruentatus, R. bilineatus*, and *R. palmarum* (Mergawy *et al*., 2011).

The overall findings of the present study are as follows: 1. The morphometric data and mating experiments provided evidence that RRPW and APW, although variable in color, are a single taxon. 2. The morphological data of Indonesian RRPW and APW color phenotypes, including RPW from the KSA, indicate that RRPW, previously considered *R. ferrugineus,* are actually color phenotypes of *R. vulneratus*. 3. In Indonesia, 32 different pronotal markings representing two palm weevil species, *R. vulneratus* (28 color phenotypes) and *R. bilineatus* (four color phenotypes), have been identified. The number of pronotal markings on male weevils was higher than that on females in both species.

ACKNOWLEDGEMENTS

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding this Research group (No: RGP-1438-009). Thanks to the Faculty of Biology, Gadjah Mada University, Indonesia, for facilitating this research. We also thank the officials of the King Saud Museum of Arthropods for use of their facility for specimen observations. We are grateful for the assistance of both Mostafa R. Sharaf, who contributed valuable input and suggestions, and Boris Kondratieff of Colorado State University, who provided a constructive review. Finally, we offer our sincere thanks for the technical support of Sri Wahyuningsih, Rahmawati, M. Syaryadhi, A. Junaedi, Supadmi, A. Priyadi, and Mr. Barjono during specimen collections in Indonesia.

REFERENCES

Abad, R. G., Bastian, J. S. A., Catiempo, R. L., Salamanes, M. L., Nemenzo-Calica, P., Rivera, W. L., 2014, Molecular profiling of different morphotypes under the genus *Rhynchophorus* (Coleoptera: Curculionidae) in Central and Southern Philippines. *Journal of Entomology and Nematology*, 6(9): 122-133.

Abraham, V. A., Al-Shuaibi, M., Faleiro, J. R., Abozuhairah, R. A., Vidyasagar, P. S. P. V., 1998, An integrated approach for the management of red palm weevil *Rhynchophorus ferrugineus* Oliv.: A key pest of date palm in the Middle East. *Sultan Qaboos University Journal for Scientific Research, Agricultural Science,* 3: 77-83.

Azmi, W. A., Zazali, C., Abdul Rahman, A.R., Azbdul Gani, N. I., 2013, A new invasive coconut pest in Malaysia: the red palm weevil (Curculionidae: *Rhynchophorus ferrugineus*). *Planter*, 89(1043): 97-110.

Bagley, M. J., Anderson, S. L, May, B., 2001, Choice of methodology for assessing genetic impacts of environmental stressors: polymorphism and reproducibility of RAPD and AFLP fingerprints. *Ecotoxicology,* 10(4): 239-244.

Barrientos, A., Barros, M. H., Valnot, I., Rotig, A., Rustin, P., Tzagoloff, A., 2002, Cytochrome oxidase in health and disease. *Gene*, 286(1): 53-64.

Branicki, W., Kupiec, T., Pawlowski, R., 2003, Validation of cytochrome b sequence analysis as a method of species identification. *Journal of Forensic Science*, 48(1): 83-87.

Bozbuga, R., Hazir, A., 2008, Pests of the palm (*Palmae* sp.) and date palm (*Phoenix dactylifera*) determined in Turkey and evaluation of red palm weevil (*Rhynchophorus ferrugineus* Olivier) (Coleoptera: Curculionidae), *EPPO Bulletin,* 38(1): 127-130.

CABI, 2015, *Rynchophorus ferrugineus* (red palm weevil). *Invasive Species Compendium Datasheet,* http://www.cabi.org/cpc/, Accessed 20 March 2016.

Carr, S. M., Marshall, H. D., 1991, Detection of intraspecific DNA sequence variation in the mitochondrial cytochrome b gene of Atlantic cod (*Gadus morhua*) by the Polymerase Chain Reaction. *Canadian Journal of Fisheries and Aquatic Sciences*, 48(1): 48-52.

Calvert L, Villarreal N, Frohlich D. 2005. Using molecular techniques to analyse whitefly species and biotypes in Latin America. *In* Jones, A. L., Markham, R.H. (ed) Whitefly and whitefly-borne viruses in the tropics: building a knowledge base for global action. p. 251-262.

Chong, J. L., H'ng, T. M., Azmi, W. A., 2015, Genetic variation and invasion history of the invasive red palm weevil (*Rhynchophorus ferrugineus* (Olivier)) in Terengganu. *International Journal of Agriculture, Forestry and Plantation*, 1: 34-43.

Collard, B. C. Y, Das, A., Virk, P. S., Mackill, D. J., 2007, Evaluation of 'quick and dirty'DNA extraction methods for marker assisted selection in rice (*Oryza sativa* L.). *Plant Breeding*, 126(1): 47-50.

DITJENBUN, 2010, Serangan kumbang merah palem *Rhynchophorus* spp., bagai api dalam sekam. Direktorat Jendral Perkebunan. Departemen Pertanian RI. http://ditjenbun.deptan.go.id/bbp2tpsur/images/stories/proteksi/rinco.pdf. Accessed on October 10, 2011. (In Indonesian).

El-Mergawy, R. A. A. M., Al Ajlan, A. M., Abdalla, N., Nasr, M. I., Silvain, J. F., 2011, Genetic comparison among *Rhynchophorus ferrugineus* and four *Rhynchophorus* species. *Journal of Agricultural Science and Technology B*, 1: 610-615.

EPPO, 2008, Data sheets on quarantine pests : *Rhynchophorus ferrugineus*. *EPPO Bulletin*, 38: 55-59.

Ernawati, F., Yuniarti, F., 2013, *Rhynchophorus* spp., kumbang yang mematikan tanaman kelapa. *Balai Besar Perbenihan dan Proteksi Tanaman Perkebunan (BBPPT). Surabaya:* 1-7. (In Indonesian).

Gadelhak, G. G., Enan, M. R., 2005, Genetic diversity among populations of red palm weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae), determined by random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR). *International Journal of Agriculture and Biology*, 7(3): 395-399.

Goodwin, T. W., 1984, *The biochemistry of carotenoids*. Vol. II. New York: Chapman & Hall.

Grether, G. F., Hudon, J., Millie, D. F., 1999, Carotenoid limitation of sexual coloration along an environmental gradient in guppies. *Proceedings of the Royal Society of London B: Biological Sciences*, 266(1426): 1317-1322.

Hairston, J. N. G., 1979, The adaptive significance of color polymorphism in two species of *Diaptomus* (Copepoda). *Limnology and Oceanography,* 24(1): 15-37.

Hall, T. A., 1999, BioEdit: a user friendly biological sequence alignment editor and analysis for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41(41): 95-98.

Hallett, R. H., Crespi, B. J., Borden, J. H., 2004, Synonymy of *Rhynchophorus ferrugineus* (Olivier), 1790 and *R. vulneratus* (Panzer), 1798 (Coleoptera, Curculionidae, Rhynchophorinae). *Journal of Natural History,* 38(22): 2863-2882.

Hallett, R. H., Gries, G., Gries, R., Borden, J. H., 1993, Aggregation pheromones of two Asian palm weevil, *Rhynchophorus ferrugineus* and *R. vulneratus*. *Naturwissenschaften,* 80: 328-331.

Harkey, G. A., Semlitsch, R. D., 1988, Effects of temperature on growth, development, and color polymorphism in the ornate chorus frog *Pseudacris ornata*, *Copeia*: 1001-1007.

Hill, G. E., Inouye, C. Y., Montgomerie, R., 2002, Dietary carotenoids predict plumage coloration in wild house finches. *Proceedings of the Royal Society of London B: Biological Sciences,* 269(1496): 1119-1124.

Hill, G. E., Montgomerie, R., 1994, Plumage colour signals nutritional condition in the house finch. *Proceedings of the Royal Society of London B: Biological Sciences,* 258(1351): 47-52.

Hoagland, K. E., 1977, A gastropod color polymorphism: one adaptive strategy of phenotypic variation. *The Biological Bulletin,* 152(3): 360-372.

Hochkirch, A., Deppermann, J., Gröning, J., 2008, Phenotypic plasticity in insects: the effects of substrate color on the coloration of two ground-hopper species. *Evolution and Development,* 10(3): 350-359.

Hsieh, H. M., Chiang, H. L., Tsai, L. C., Lai, S. Y., Huang, N. E., Linacre, A., Lee, J. C. I., 2001, Cytochrome b gene for species identification of the conservation animals. *Forensic Science International*, 122(1): 7-18.

Jones, C. J., Edwards, K. J., Castaglione, S., Winfield, M. O., Sala, F., Van de Wiel, C., Bredemeijer, G., Vosman, B., Matthes, M., Daly, A., Brettschneider, R., Bettini, P., Buiatti, M., Maestri, E., Malcevschi, A., Marmiroli, N., Aert, R., Volckaert, G., Rueda, J., Linacero, R., Vazquez, A., Karp A., 1997, Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Molecular breeding,* 3(5): 381-390.

Kaakeh, W., Abou-Nour, M. M., Khamis, A. A., 2001, Mass rearing of red palm weevil, *Rhynchophorus ferrugineus* Oliv., on sugarcane and artificial diets for laboratory studies : *Illustration of methodology proceedings of the second international conference on date palm, Al-Ain, UAE:* 344-357.

Kalshoven, L. G. E., 1981, *Pest of crops in Indonesia*. P. T. Ichtiar Baru - Van Hoeve. Jakarta.

Kurose, N., Abramov, A. V., Masuda, R., 2000, Intrageneric diversity of the cytochrome b gene and phylogeny of Eurasian species of the genus *Mustela* (Mustelidae, Carnivora). *Zoological Science*, 17(5): 673-679.

Lohr, B., Vásquez-Ordóñez, A. A., Lopez-Lavalle, L. A. B., 2015, *Rhynchophorus palmarum* in disguise: Undescribed polymorphism in the “black” palm weevil. *PloS one,* 10(12): 1-14.

Malumphy, C., Moran, H., 2007, Plant pest notice: red palm weevil, *Rhynchophorus ferrugineus*. *Department for Environment, Food and Rural Affairs, Central Science Laboratory*.

McGraw, K. J., Ardia, D. R., 2003, Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. *The American Naturalist,* 162(6): 704-712.

Micheli, M. R., Bova, R., Pascale, E., D’Ambrosio, E., 1994, Reproducible DNA fingerprinting with the random amplified polymorphic DNA (RAPD) method. *Nucleic Acids Research*, 22(10): 1921-1922.

Mizzi S., Dandria D., Mifsud D., Longo S, 2009, The red palm weevil, *Rhynchophorus ferrugineus* (Olivier, 1790) in Malta (Coleoptera: Curculionoidea). *Bulletin of the Entomological Society of Malta,* 2: 111-121.

Nadiah, A., 2016, Si kumbang, penyebab matinya tanaman kelapa. Artikel DITJENBUN Pertanian. Surabaya. http://balaisurabaya.ditjenbun.pertanian.go.id. Accesed March 12th, 2018.

Parkash, R., Singh, S., Ramniwas, S., 2009, Seasonal changes in humidity level in the tropics impact body color polymorphism and desiccation resistance in *Drosophila jambulina*-Evidence for melanism-desiccation hypothesis. *Journal of insect physiology,* 55(4): 358-368.

Pelikh, K., 2009, First report of RPW in Republic of Georgia. *Red Palm Weevil* http://www.redpalmweevil.com/newlook/RPWReport/Georgia.html, Accessed May 21st, 2011.

Penner, G. A., Bush, A., Wise, R., Kim, W., Domier, L., Kasha, K., Laroche, A., Scoles, G., Molnar, S. J., Fedak, G., 1993, Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. *Genome Research,* 2(4): 341-345.

Pracaya, 1991, *Hama dan penyakit tanaman*. Penebar Swadaya. Jakarta (In Indonesian), 221-224

Prasetyo, A. E., Susanto, A., Utomo, C., Herawan, T., 2009, Sinergisme dua feromon agregat dalam pengendalian *Oryctes rhinocheros* dan *Rhynchophorus* spp. di perkebunan kelapa sawit. *Jurnal Penelitian Kelapa Sawit,* 17(1): 23-29. (In Indonesian with English abstract)

Ratmawati, I., 2015, Tingkat serangan hama tanaman kelapa di Kabupaten Probolinggo pada bulan Mei 2015. *Buletin DISHUTBUN Probolinggo*: 1-7.

Rugman-Jones, P. F., Hoddle, C. D., Hoddle, M. S., Stouthamer, R., 2013, The lesser of two weevils: molecular-genetics of pest palm weevil populations confirm *Rhynchophorus vulneratus* (Panzer 1798) as a valid species distinct from *R. ferrugineu*s (Olivier 1790), and reveal the global extent of both. *PloS one,* 8(10): e78379.

Santosa, I. B., 2016, *Rhynchophorus* spp., kumbang yang mematikan tanaman kelapa. Artikel http://pertanian.jombangkab.go.id. Accessed March 12th, 2018.

Sánchez-Ruiz, M., Sanmartín, I., 2000, Separation of *Aspidiotes* species using morphometric analysis (Coleoptera: Curculionidae). *European Journal of Entomology,* 97(1): 85-94.

Shahina, F., Salma, J., Mehreen, G., Bhatti, M., Tabassum, K., 2009, Rearing of *Rhynchophorus ferrugineus* in laboratory and field conditions for carrying out various efficacy studies using EPNs. *Pakistan Journal of Nematology,* 27(2): 219-228.

Skroch, P., Nienhuis, J., 1995, Impact of scoring error and reproducibility RAPD data on RAPD based estimates of genetic distance. *Theoretical and Applied Genetics,* 91(6-7): 1086-1091.

Sneath, P. H. A, Sokal, R. R., 1973, Numerical taxonomy : The principles and practice of numerical classification. W. H. Freeman and Company, San Fransisco. 573pp.

Soroker, V., Blumberg, D., Haberman, A., Hamburger-Rishard, M., Reneh, S., Talebaev, S., Harari, A. R., 2005, Current status of red palm weevil infestation in date palm plantations in Israel. *Phytoparasitica,* 33(1): 97-106.

SPSSInc, 2005, SPSS for Windows user's guide. *Version 13.0*.

Sword, G. A., Simpson, S. J., El Hadi, O. T. M., Wilps, H., 2000, Density–dependent aposematism in the desert locust. *Proceedings of the Royal Society of London B: Biological Sciences,* 267(1438): 63-68.

Tambe, J. T., Riolo, P., Okolle, J. N., Isidoro, N., Fanciulli, P. P., Dallai, R., 2013, Sexual size differences and colour polymorphism of *Rhynchophorus phoenicis* in the southwest region of Cameroon. *Bulletin of Insectology,* 66: 153-159.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013, MEGA6: Molecular evolutionary genetic analysis version 6.0. *Molecular Biology and Evolution*, 30(12): 2725-2729

Théry, M., Pincebourde, S., Feer, F., 2008, Dusk light environment optimizes visual perception of conspecifics in a crepuscular horned beetle. *Behavioral Ecology,* 19(3): 627-634.

Trisnadi, R., 2014, Kumbang sagu (*Rhynchophorus* sp.) penyebab kematian tanaman kelapa. *Artikel Dinas Kehutanan dan Perkebunan Kabupaten Probolinggo:* 1- 6. (In Indonesian)

Turner, J. R. G., 1968, Some new *Heliconius* pupae: their taxonomic and evolutionary significance in relation to mimicry (Lepidoptera, Nymphalidae). *Journal of Zoology,* 155(3): 311-325.

USDA-CPHST, 2010, Host map *Rhynchophorus ferrugineus*, red palm weevil. *United Stated Department of Agriculture, Animal and Plant Health Inspection Service, Plant Protection and Quarantine, Center for Plant Health Science and Technology. NSCU APHIS Plant Pest Forecasting System*, http://www.nappfast.org/caps_pests/CAPS_Top_50.htm. Accessed on May 23, 2010.

Vanderplank, F. L., 1960, The bionomics and ecology of the red tree ant, *Oecophylla* sp., and its relationship to the coconut bug *Pseudotheraptus wayi* Brown (Coreidae). *The Journal of Animal Ecology*: 15-33.

Verma, S. K., Singh, L., 2002, Novel universal primers established identification of an enermous animal species for forensic application. *Molecular Ecology Notes*, 3(1): 28-31.

Wattanapongsiri, A., 1966, A revision of the genera *Rhynchophorus* and *Dynamis* (Coleoptera: Curculionidae). *Department of Agriculture Science Bulletin,* Bangkok. Thailand, 1(1): 418.

Wibowo, E., Ernawati, D., 2013, Perkembangan serangan hama *Rhynchophorus* spp. pada tanaman kelapa di Propinsi Jawa Timur periode bulan September 2012. *Buletin BBPPT Surabaya DITJENBUN Pertanian:* 1-8. (In Indonesian)

Yamasaki, A., Shimizu, K., Eujisaki, K., 2009, Effect of host plant part on larval body-color polymorphism in *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Annals of the Entomological Society of America,* 102(1): 76-84.

Yong, K. W., 2016, Morphological, Genetic and Biological Studies of Red Palm Weevil, *Rhynchophorus* spp. (Coleoptera: Curculionidae) in Terengganu, Malaysia. MSc thesis. Universiti Malaysia Terengganu, Terengganu, Malaysia. 95 pp.

Yuliyanto, Y., Ernawati, D., 2015, Serangan *Rhynchophorus ferrugineus* di wilayah Jawa Timur. *Buletin DITJENBUN Pertanian. BBPPT Surabaya:* 1-7. (In Indonesian).

Received: January 26, 2017 *Accepted: September 17, 2018*