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Undescribed color polymorphism of the Asiatic palm weevil, *Rhynchophorus vulneratus* Panzer (Coleoptera: Curculionidae) in Indonesia: biodiversity study based on COI gene

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Abstract

Palm weevils are notorious insect pests of coconut palms in Indonesia and other palm species worldwide. Indonesian palm weevils exhibiting a rusty red marking on the pronotum are classified as the red palm weevil (*Rhynchophorus ferrugineus* Olivier; Coleoptera: Dryophthoridae). However, morphology-based identification and crossbreeding studies suggested that these insects are color morphs of the Asiatic palm weevil (*Rhynchophorus vulneratus* Panzer; Coleoptera: Dryophthoridae). The purpose of this study was to clarify the presence of undescribed color polymorphisms of the Asiatic palm weevil from Indonesia using a partial sequence of the cytochrome oxidase subunit I (COI) gene. The COIs of 107 specimens of palm weevils collected from Indonesia, Saudi Arabia, and Pakistan were amplified using Folmer primers. COI analysis revealed high intraspecific variability in palm weevils from Indonesia and Saudi Arabia. Although Folmer primers of COI are powerful species identification tools in many taxa, they were unsuitable in this research for Indonesian palm weevils because interspecific variation was lower than intraspecific variation. This study concluded that undescribed color polymorphisms exist in the Asiatic palm weevil. The rusty red polymorphisms of the Asian palm weevil might be erroneously identified as *R. ferrugineus*.

Key Words: COI; diversity; DNA barcoding; mitochondrial DNA; red palm weevil

Resumen

Los gorgojos (o picudos) de las palmeras son plagas notorias de las palmas de coco en Indonesia y otras especies de palmeras en todo el mundo. Los gorgojos de la palma de Indonesia que exhiben una marca roja oxidada en el pronoto se clasifican como el picudo rojo de la palma (*Rhynchophorus ferrugineus* Olivier; Coleoptera: Dryophthoridae). Sin embargo, identificaciones basadas en la morfología y los estudios de cruzamiento sugirieron que estos insectos son morfos de color del picudo asiático (*Rhynchophorus vulneratus* Panzer; Coleoptera: Dryophthoridae). El propósito de este estudio fue aclarar la presencia de polimorfismos de color no descritos del gorgojo asiático de la palma de Indonesia utilizando una secuencia parcial del gen de la subunidad I (COI) de la citocromo oxidasa. La COI de 107 especímenes de gorgojos de la palma recolectados de Indonesia, Arabia Saudita y Pakistán se amplificaron utilizando cebadores Folmer. El análisis de COI reveló una alta variabilidad intraespecífica en los gorgojos de la palma de Indonesia y Arabia Saudita. Aunque los primers de Folmer de COI son poderosas herramientas de identificación de especies en muchos taxones, no fueron adecuados en esta investigación para los gorgojos de la palma de Indonesia porque la variación interespecífica fue menor que la variación intraespecífica. Este estudio concluyó que existen polimorfismos de color no descritos en el gorgojo asiático de la palma. Los polimorfismos rojos oxidados del picudo asiático de la palmera podrían identificarse erróneamente como *R. ferrugineus*.

Palabras Clave: COI; diversidad; códigos de barras de ADN; ADN mitocondrial; picudo rojo

Palm weevils, *Rhynchophorus* spp. (Coleoptera: Curculionidae), are the second-most damaging insect pests on coconut palm, *Cocos nucifera* Linnaeus (Arecaceae) in Indonesia (Ernawati & Yuniarti 2013; Trisnadi 2014; Ratmawati 2015; Yulianto & Ernawati 2015). They also damage the sago palm (*Metroxylon sagu* Rottb.; Arecaceae), the solitary sugar palm (*Arenga pinnata* (Wurmb) Merrill.; Arecaceae), the oil palm (*Elaeis guineensis* Jacq.; Arecaceae), the large-leaved palm (*Corypha gebanga* Zelfst.; Arecaceae), the silver date palm (*Phoenix sylvestris* (L.)Roxb.; Arecaceae), and the African fan palm (*Borassus flabellifer* L.; Arecaceae) (Leefmans 1920). Severe infestation of coconut palm has been reported recently in East Java, which includes the districts of Ponorogo, Kediri, Jombang, and Probolinggo (Ernawati & Yuniarti 2013; Trisnadi 2014; Ratmawati 2015; Yulianto & Ernawati 2015).

Changes were made to the classification of weevil species in Indonesia. Three palm weevil species were previously identified: the red palm weevil (*Rhynchophorus ferrugineus* Olivier; Coleoptera: Dryophthoridae), the Asiatic palm weevil (*R. vulneratus* Panzer; Coleoptera: Dryophthoridae), and the black palm weevil (*R. bilineatus* Montrouz-

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ier; Coleoptera: Dryophthoridae) (Wattanapongsiri 1966; Kalshoven 1981; Pracaya 1991). The red palm weevil was found in Java, whereas the Asiatic palm weevil and the black palm weevil were found in Sumatra and the eastern regions of Indonesia (Moluccas and Papua islands), respectively (Kalshoven 1981).

The morphological identification of the Indonesian palm weevils from Sumatra, Java, Madura, Bali, Sulawesi, and West Papua islands was reported by Sukirno et al. (2015). Twenty morphs of rusty red Asiatic palm weevil, 7 morphs of red stripe Asiatic palm weevil, 1 morph of intermediate palm weevil, and 4 morphs of black palm weevil were identified. The intermediate palm weevil specimens exhibited combinations of rusty red, much like red palm weevil, with a red stripe on the pronotum. Most of the rusty red Asiatic palm weevil and red stripe Asiatic palm weevil morphs coexist in the same colonies within the same host plants (Hallet et al. 2004; Sukirno et al. 2015). On the basis of morphometric analysis and mating experiments for up to 3 generations, these weevils were considered color-polymorphic *R. vulneratus*.

Unambiguous species identification is critical for pest control. The identification of color-polymorphic species, as in the case of palm weevils in this study, requires molecular approaches for confirming morphology-based identification. For example, cytochrome oxidase subunit I (COI) was used for the identification of palm weevils from 23 countries (Rugman-Jones et al. 2013), including southern and central Philippines (Abad et al. 2014). It also was used for the prediction of red palm weevil invasion in the Middle East and the Mediterranean basin (El-Mergawy et al. 2011a). Meanwhile, cytochrome b (CyB) and internal transcribed spacer ribosomal DNA (ITS-rDNA) markers have been used to reveal red palm weevil diversity in the Mediterranean Basin and Saudi Arabia (El-Mergawy et al. 2011b). Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) has been carried out on the red palm weevil in the United Arab Emirates (Gadelhak & Enan 2005) and in 13 countries, including the Middle East, the Mediterranean Basin, and South Asia (El-Mergawy et al. 2011c).

The COI marker has been used in other insect species; for example, Grapputo et al. (2005) used it to reveal the invasion history of the North American and European Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), and McKenzie et al. (2009) used it on the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) in Florida. Moreover, it has been applied in the identification of Australian fishes (Ward et al. 2005), North American birds (Kerr et al. 2007), longicorn beetles (Coleoptera: Cerambycidae) (Nakamine & Takeda 2008), whiteflies (Dinsdale et al. 2010), and ambrosia beetles (Coleoptera: Scolytinae: Platypodinae) (Chang et al. 2013). Some accuracy limitations of DNA barcoding for species identification have been reported in Diptera (Meier et al. 2006) and blue lycaenid butterflies (Wiemers & Fiedler 2007). Nevertheless, the COI marker remains a powerful tool for revealing species diversity, especially when working with degraded DNA material (Hajibabaei et al. 2006; Meusnier et al. 2008). It also has been widely used to complement morphology-based identification (Hajibabaei et al. 2007; Goldstein & DeSalle 2011), particularly for cryptic species (Hebert et al. 2004).

This study was designed to clarify palm weevil diversity on the 6 principal islands of Indonesia using the COI marker, and to evaluate its suitability for species-level identification. Our results served as a test of previous morphology-based identifications and documented the identity of Indonesian palm weevils. *Rhynchophorus ferrugineus* collected from Saudi Arabia and Pakistan were used for further confirmation. We hypothesized that the Asiatic palm weevil from Indonesia has undescribed "rusty red" polymorphisms that might cause it to be erroneously identified as *R. ferrugineus*.

Materials and Methods

PALM WEEVIL SURVEY AND COLLECTION

Palm weevils were collected from 23 localities on 6 islands in Indonesia, covering 7 provinces (Table 1): Aceh Special Region (Sumatra island), Central Java (Java island), Jogjakarta Special Region (Java island), East Java (Java and Madura islands), Bali (Bali island), Gorontalo (Sulawesi island), and Sorong (West Papua island). The survey and collection were conducted between 2012 and 2014. Two populations of the red palm weevil in Alamariyah, the Riyadh Province of Saudi Arabia, and 4 populations from Khudai, the Punjab Province of Pakistan, were used for the comparisons. The weevils were preserved in 96% ethanol at 4 °C until further use.

The weevils were separated into the following categories based on their pronotum color pattern: the Asiatic palm weevil with rusty red morphs, the Asiatic palm weevil with red stripe morphs, the Asiatic palm weevil with intermediate morphs between rusty red and red stripe morphs, and the black palm weevil with or without longitudinal lines. Voucher specimens were deposited at the King Saud University Museum of Arthropods, Riyadh, Kingdom of Saudi Arabia.

TISSUE COLLECTION

At least 3 individuals of each pronotal-marking category from each locality were used for the study. Approximately 2 mm^3 of pronotal muscle tissue was taken by pulling it from the posterior side of the pronotum using sterilized fine forceps. The tissue was then placed into a 200 µL thin-wall PCR tube and dried in a vacuum rotary drier (Concentrator Plus, Eppendorf AG, Hamburg, Germany) at 45 °C for 10 min to remove the ethanol residue.

CRUDE MITOCHONDRIAL DNA PREPARATION FOR COI ANALYSIS

A modified alkaline lysis method was used to extract mitochondrial DNA from the muscle tissue samples for COI gene analysis (Wang et al. 1993; Collard et al. 2007; Wang et al. 2009). Approximately 25 µL of 50 mM NaOH solution was added to dried muscle tissue in thin-wall 200 µL PCR tubes, vortexed vigorously for 10 s, and spun down in a microcentrifuge (IKA® mini G IKA®–Werke GmbH and Co. KG, Staufen im Breisgau, Germany). The sample was then heated to 95 °C for 20 min in a PCR machine (GeneAmp® PCR System 9700, Applied Biosystem, Carlsbad, California, USA). The extract was left for 5 min at ambient temperature, followed by homogenization for 10 s using a vortex. About 25 µL of 200 mM Tris-HCl (pH 8.0) was added to each sample and then homogenized for 5 s using a vortex. The debris was precipitated in a centrifuge for 1 min at 10,000 rpm and 25 °C. Subsequently, 3 µL of the supernatant was used as the PCR template to amplify the COI gene region. The remaining extracts were stored at −20 °C for future use.

COI GENE AMPLIFICATION

The COI gene was amplified in 30 µL of the KOD FX Neo polymerase kit solution (Toyobo Co., LTD, Osaka, Japan) that contained 0.2 µM each of LCO 1490 and HCO 2198 primers (Folmer et al. 1994) synthesized by IDT DNA technologies (IDT DNA, Leuven, Belgium) and 3 µL of crude DNA templates. DNA amplification was carried out in a thermocycler (GeneAmp® PCR System 9700 Applied Biosystem, Staufen im Breisgau, Germany) with a heated lid. The amplification protocol was as follows: initial denaturation by heating at 95 °C for 2 min; 35 amplification cycles of 98 °C for 10 s, 48 °C for 30 s, and 68 °C for 40 s; and final extension at 68 °C for 5 min, followed by cooling down to 4 °C for an infinite time.

Table 1. Localities (latitude and longitude) of palm weevil collections covering 7 provinces in Indonesia and several locations in Saudi Arabia and Pakistan.

No.	Locality, Province*	Code	Latitude	Longitude	Species
1.	Bireun, Aceh IN	BIR A	5.1953 N	96.7107 E	*R. vulneratus*
2.	Lambrita, Aceh IN	LAM A	5.5763 N	95.3871 E	*R. vulneratus*
3.	Katiasa, Bali IN	BAL A	8.1608 S	115.1387 E	*R. bilineatus*
4.	Bumi Ayu, Central Java IN	BAY A	7.5381 S	109.1794 E	*R. vulneratus*
5.	Jetis A, Central Java IN	JTS A	7.6684 S	110.4987 E	*R. vulneratus*
6.	Jetis B, Central Java IN	JTS B	7.6678 S	110.4967 E	*R. vulneratus*
7.	Jetis C, Central Java IN	JTS C	7.6678 S	110.4965 E	*R. vulneratus*
8.	Jetis D, Central Java IN	JTS D	7.6686 S	110.4988 E	*R. vulneratus*
9.	Jetis E, Central Java IN	JTS E	7.6685 S	110.4973 E	*R. vulneratus*
10.	Kebon Alas A, Central Java IN	KBN A	7.6865 S	110.4885 E	*R. vulneratus*
11.	Kebon Alas B, Central Java IN	KBN B	7.6869 S	110.4885 E	*R. vulneratus*
12.	Kebon Alas C, Central Java IN	KBN C	7.6871 S	110.4884 E	*R. vulneratus*
13.	Gondang, Central Java IN	GDG A	7.6753 S	110.4967 E	*R. vulneratus*
14.	Pemalang, Central Java IN	PEM A	6.8598 S	109.5075 E	*R. vulneratus*
15.	Madura A, East Java IN	MAD A	6.9446 S	113.5491 E	*R. vulneratus*
16.	Madura B, East Java IN	MAD B	6.9352 S	113.5553 E	*R. vulneratus*
17.	Sumalata, Gorontalo, Sulawesi IN	SUM A	0.9746 N	122.5041 E	*R. vulneratus*
18.	Aitinyo, West Papua IN	AIT A	1.4608 S	132.0229 E	*R. bilineatus*
19.	Moswaren, West Papua IN	MOS A	1.5074 S	132.2330 E	*R. bilineatus*
20.	Teminabuan, West Papua IN	PAP A	1.4491 S	132.0236 E	*R. bilineatus*
21.	Pendowoharjo, Jogjakarta IN	PND A	7.6841 S	110.4341 E	*R. vulneratus*
22.	Kuwung A, Jogjakarta IN	KUW A	7.6737 S	110.4581 E	*R. vulneratus*
23.	Kuwung B, Jogjakarta IN	KUW B	7.6739 S	110.4581 E	*R. vulneratus*
24.	Alamariyah A, Riyadh SA	AMR A	24.8045 N	46.5087 E	*R. ferrugineus*
25.	Alamariyah B, Riyadh SA	AMR B	24.8175 N	46.5140 E	*R. ferrugineus*
26.	Khudai A, Punjab PK	KUD PK1	30.0594 N	71.1794 E	*R. ferrugineus*
27.	Khudai B, Punjab PK	KUD PK2	30.0594 N	71.1794 E	*R. ferrugineus*
28.	Khudai C, Punjab PK	KUD PK3	30.0594 N	71.1794 E	*R. ferrugineus*
29.	Muzafargarh, Punjab PK	MZF PK1	30.0594 N	71.1794 E	*R. ferrugineus*

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

AMPLICON ANALYSIS

About 1 µL each of unpurified COI 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, Tokyo, Japan). The 1 kb Plus DNA Ladder (Invitrogen Life Technologies, Waltham, Massachusetts, USA) was used as a marker. The gels then were placed in ethidium bromide solution (4 µL per 200 mL of 1 × TAE). Staining was performed by gentle agitation and shaking for 30 min at 45 rpm on an orbital shaker (Orbital incubator SI 500 Stuart®, Bibby Scientific Ltd., Stone, Staffordshire, United Kingdom). The stained gels were visualized under UV light in a gel documentation system (BioDocAnalyze, Biometra, Gottingen, Denmark). The amplified PCR products of COI (about 700 bp) were sent for sequencing in both directions by using the Sanger method (BGI, Hong Kong, China).

COI SEQUENCE ANALYSES

Both directions of the COI sequences aligned separately in BioEdit ver. 7.2.2 (Hall 1999). The primers were trimmed to obtain 657 bp nucleotides (nt). Basic local alignment search tool of the nucleotides was carried out using NCBI GenBank databases to confirm the COI sequences obtained. The validated sequences then were aligned using Clustal*W* multiple alignments in the BioEdit program (Hall 1999). The Kimura 2-parameter model available in MEGA 6.06 (Tamura et al. 2013) was used to estimate the pattern and rate of substitution, transition/transversion bias, and genetic diversity. This model also was employed to construct the neighbor joining phylogeny (Tamura et al. 2013) using 1,000 bootstrap values. The transition and transversion substitutions were included in the analysis. Gaps or missing data were treated as complete deletions.

Results

The diversity of palm weevils representing 23 localities in Indonesia, 2 localities in Saudi Arabia, and 4 localities in Pakistan was identified using the COI marker. A total of 107 clean sequences were obtained from these localities. The comparison showed no insertion or deletion in any of the obtained sequences. The approximate transition and transversion bias (R) was 1.22. The relative frequencies of A, T, C, and G were 0.27, 0.34, 0.21, and 0.17, respectively. The transition and transversion substitution rates were 13.76 and 5.62, respectively. The present study revealed 5 conserved regions: TATACTTTATTTTTGG (8–25 nt), ATTATAATTTTTTTTTATA (160–177 nt), GCAGGAACAGGTTGAACAGT (344–363 nt), TCTGTAGATT TAGCTATTTTTA-G (409–432 nt), and TATTAACTGACCGAAAT AT (619–637 nt). All the sequences have been deposited in the Barcode of Life Data System (BOLD) with accession numbers PWINA001-16 to PWINA064-16, PWINA086-16 to PWINA118, and PWINA122-16 to PWINA132-16 (accessible at http://boldsystems.org/).

A high variability in the COI gene within the populations of palm weevils analyzed in this study was revealed (Table 2). The majority of the weevils exhibited non-identical sequences regardless of the level of similarity in their pronotal phenotypes. Only 2 of the 107 sequences (1.9%) were identical. The multiple alignments showed that COI gene diversity within black palm weevil samples from Bali was the highest (d

Table 2. Palm weevils collected from Indonesia, Saudi Arabia, and Pakistan and their genetic diversity within the populations on the basis of COI gene analysis.

No.	Locality/ Province*	N	Code	Species	COI[a]
1.	Bireun, Aceh IN	3	BIR A	R. vulneratus	0.001
2.	Lambrita, Aceh IN	4	LAM A	R. vulneratus	0.001
3.	Katiasa, Bali IN	6	BAL A	R. bilineatus	0.710
4.	Bumi Ayu, Central Java IN	2	BAY A	R. vulneratus	0.007
5.	Jetis A, Central Java IN	2	JTS A	R. vulneratus	0.003
6.	Jetis B, Central Java IN	5	JTS B	R. vulneratus	0.001
7.	Jetis C, Central Java IN	1	JTS C	R. vulneratus	na
8.	Jetis D, Central Java IN	7	JTS D	R. vulneratus	0.012
9.	Jetis E, Central Java IN	4	JTS E	R. vulneratus	0.222
10.	Kebon Alas A, Central Java IN	10	KBN A	R. vulneratus	0.009
11.	Kebon Alas B, Central Java IN	3	KBN B	R. vulneratus	0.242
12.	Kebon Alas C, Central Java IN	4	KBN C	R. vulneratus	0.004
13.	Gondang, Central Java IN	3	GDG A	R. vulneratus	0.003
14.	Pemalang, Central Java IN	3	PEM A	R. vulneratus	0.013
15.	Madura A, East Java IN	4	MAD A	R. vulneratus	0.022
16.	Madura B, East Java IN	4	MAD B	R. vulneratus	0.025
17.	Sumalata, Gorontalo, Sulawesi IN	6	SUM A	R. vulneratus	0.001
18.	Aitinyo, West Papua IN	2	AIT A	R. bilineatus	0.141
19.	Moswaren, West Papua IN	1	MOS A	R. bilineatus	na
20.	Teminabuan, West Papua IN	1	PAP A	R. bilineatus	na
21.	Pendowoharjo, Jogjakarta IN	1	PND A	R. vulneratus	na
22.	Kuwung A, Jogjakarta IN	10	KUW A	R. vulneratus	0.003
23.	Kuwung B, Jogjakarta IN	10	KUW B	R. vulneratus	0.148
24.	Alamariyah A, Riyadh SA	2	AMR A	R. ferrugineus	< 0.001
25.	Alamariyah B, Riyadh SA	3	AMR B	R. ferrugineus	0.001
26.	Khudai A, Punjab PK	2	KUD PK1	R. ferrugineus	0.002
27.	Khudai B, Punjab PK	2	KUD PK2	R. ferrugineus	0.002
28.	Khudai C, Punjab PK	2	KUD PK3	R. ferrugineus	0.002
29.	Muzafargarh, Punjab PK	3	MZF PK1	R. ferrugineus	0.001

Note: *IN: Samples from Indonesia; PK: Samples from Pakistan; SA: Samples from Saudi Arabia. N indicates the number of individuals used in the analysis. Numbers in column COIa indicate the genetic diversity within the population; na: no available data because only one sequence was available.

= 0.71), followed by the samples from Kebonalas B (d = 0.24). By contrast, COI genetic diversity in the samples of *R. ferrugineus* from Saudi Arabia and Pakistan was very low (< 0.001 and < 0.002, respectively). The genetic distance between populations representative of Saudi Arabia and Pakistan was 0.002 and 0.003, respectively. The overall genetic diversity of the COI gene within populations of *R. vulneratus*, *R. bilineatus*, and *R. ferrugineus* was higher than that between populations. On the basis of nucleotide diversity as measured by using the Kimura 2-parameter model, the average intrapopulation and interpopulation genetic diversities were 0.108 and 0.016, respectively.

The neighbor joining phylogenetic analysis of palm weevil populations (Table 1) comprised 105 haplotypes and revealed 3 lineages, which were designated as C_A, C_B, and C_C (Fig. 1). Lineage C_A was represented by a single sample of the black palm weevil (*R. bilineatus*) from Bali. Lineage C_B composed Asiatic palm weevils with rusty red morphs from Kuwung B, Jetis E, and Madura A; the black palm weevil from Teminabuan and Aitinyo; and the Asiatic palm weevil with red stripes from Pemalang. Lineage C_C was composed of Asiatic palm weevils with rusty red morphs, Asiatic palm weevil with red stripes, black palm weevil, and Asiatic palm weevil with morphs intermediate between rusty red and red stripe from Indonesia, and red palm weevil from Saudi Arabia and Pakistan.

Lineages C_B and C_C could be separated into sub-clusters. Lineage C_B was separated into 2 sub-clusters: SC_{B1}, that comprised Asiatic palm weevils with rusty red morphs from Kuwung and Jetis, and black palm weevil from Teminabuan and Aitinyo; and SC_{B2} consisted of Asiatic palm weevil with red stripes from Pemalang, and Asiatic palm weevils with rusty red morphs from Madura. Lineage C_C was separated into 48 sub-clusters. All red palm weevils from Pakistan were grouped into a single sub-cluster within lineage C_C. This sub-cluster could be further separated into subgroups: subgroup Muzafargarh and Khudai (localities 1, 2, and 3) and subgroup Khudai (localities 1 and 2; Fig. 1). By contrast, the red palm weevils from Saudi Arabia were not clustered.

Discussion

The Indonesian Ministry of Agriculture considers palm weevils (*Rhynchophorus* sp.) to be one of the most serious insect pests of coconut palms in Indonesia, especially in Java (Ernawati & Yuniarti 2013; Wibowo & Ernawati 2013; Trisnadi 2014; Ratmawati 2015; Yulianto & Ernawati 2015). These insects also have high potential as pests of oil palm plantations in Sumatra (Prasetyo et al. 2009). Yuliyanto and Ernawati (2015) estimated that *Rhynchophorus* has infested 2,340 ha of coconut plantations in East Java, and 200 ha have been treated using pheromone traps. Their results showed that palm weevils are the second most damaging pest of coconut palms, after the rhinoceros beetle, *Oryctes rhinoceros* (L.) (Coleoptera: Scarabaeidae).

Neighbor joining analysis based on the COI marker revealed that palm weevils' diversity was high. The Asiatic palm weevil with rusty red morphs, the Asiatic palm weevil with intermediate between rusty red and red stripe morphs, and the *R. bilineatus* from Indonesia, as well as *R. ferrugineus* from Saudi Arabia, were overlaps (Fig. 1). There were high genetic diversity in *R. bilineatus* from Bali (d = 0.72) and West Papua (d = 1.0), as well as the

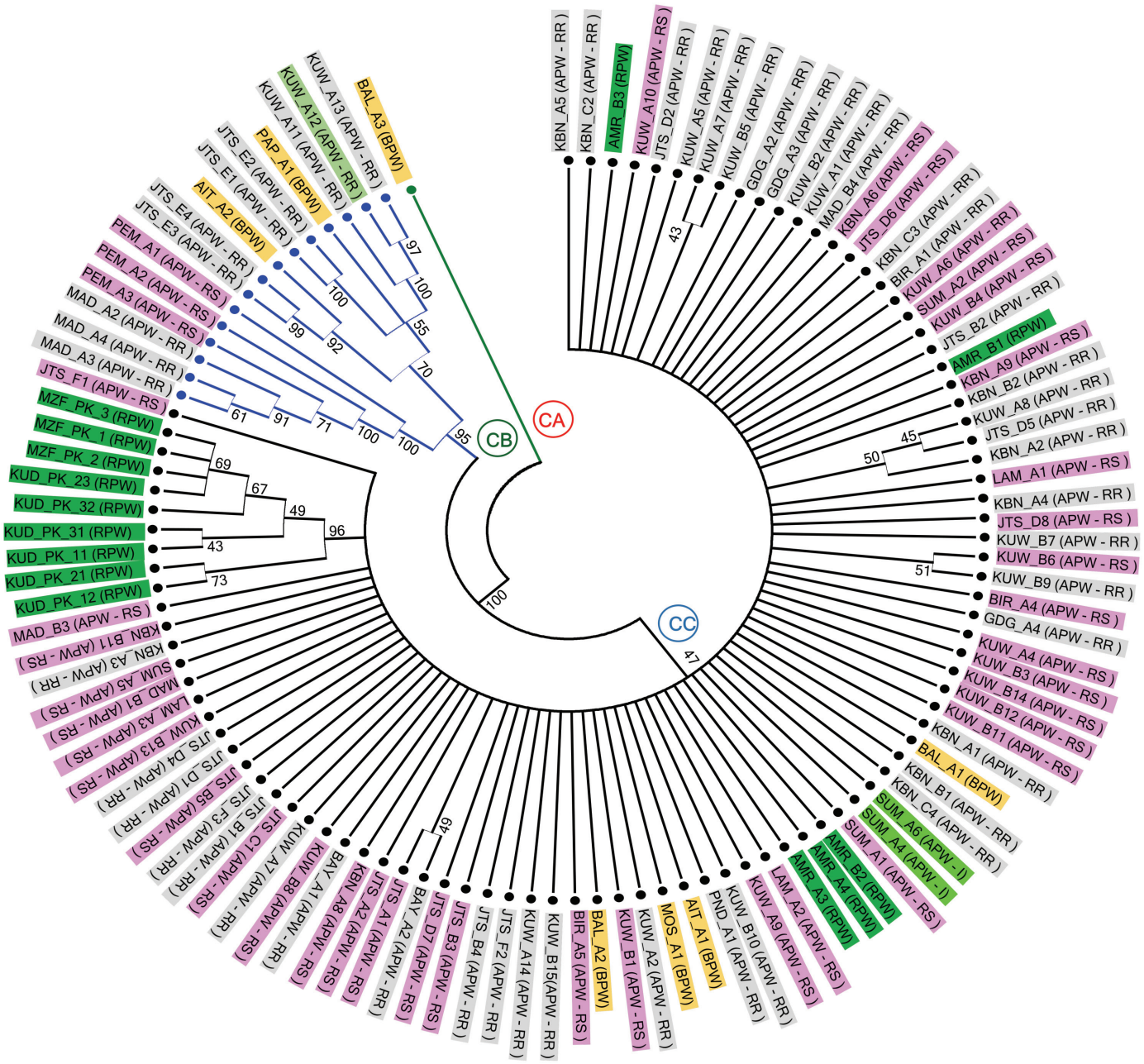

Fig. 1. Genealogical relationship of 105 sequence haplotypes of palm weevils collected from Indonesia and *Rhynchophorus ferrugineus* from Saudi Arabia and Pakistan based on the cytochrome oxidase subunit I (COI) gene (657 bp) using the neighbor joining method with 1,000 bootstraps. The colors indicate the palm weevil color morphs or species: - Asiatic palm weevil with rusty red morphs (APW–RR); - Asiatic red palm weevil with red stripe morphs (APW–RS); - Asiatic palm weevil with intermediate color between rusty red and red stripe morphs (APW–I); - black palm weevil (BPW as an outgroup); and - red palm weevil, *R. ferrugineus* (RPW). The numbers at the branching points indicate the bootstrap values. CA = Lineage A, CB = Lineage B, CC = Lineage C, SCA = Sub-cluster A, SCB = Sub-cluster B.

R. vulneratus samples from East Java (d = 1.3). Indonesia is known to be one of the mega biodiversity countries in the world (Brooks et al. 2006). Its climatic conditions are favorable for the growth of diverse organisms, including palm weevils. In this case, palm weevils lack attention, and no significant treatment of the infested palms has been made, thereby allowing the weevils to spread freely, disperse, and interbreed among populations. Inside an infested coconut tree, hundreds of weevils in different stages may be found. To date, most of the region's farmers still ignore the infested palms. A few decades ago, the grubs of weevils were considered edible insects (Ramandey & van Mastrigt 2010), but their popularity has declined. This scenario may have caused the weevils' population to increase. This high population density may have become the principal inducer of high diversity (Amos & Harwood 1998).

Neighbor joining analysis showed that red palm weevil populations from Saudi Arabia were not clustered, in contrast to those from Pakistan. In Saudi Arabia, a high frequency of date palm transportation was reported in the past few decades (Ministry of Agriculture 2014, personal communication), which possibly facilitated red palm weevil dispersal and interbreeding. By contrast, red palm weevil populations in Pakistan were isolated in several areas (Sind and Punjab), and only a few reports regarding this topic have been published. In 2009, 5.8% of the date palms in Sind Province were infested by red palm weevil

(Abul-Soad et al. 2015). Furthermore, the GenBank database (http://www.ncbi.nlm.nih.gov/nuccore, accessed in Aug 2016) indicated that some red palm weevils collected from the Punjab province have been used for molecular studies.

In this study, that involved 23 localities in Indonesia, the use of Folmer primers revealed 96 haplotypes from 96 specimens (100%). Rugman-Jones et al. (2013) used several primer combinations for the DNA barcoding of *R. vulneratus* from Indonesia. They collected from 7 palm weevil localities: 3 localities from Java, 3 localities from Sumatra, and 1 locality from Bali. In 107 sequences (528 bp), they identified 50 haplotypes (46.7%). The use of short partial sequences (220 bp) of COI for the identification of 2 palm weevil phenotypes in the Philippines implied that the phenotypes share high similarity (Abad et al. 2014). Our result was highly different than those previous reports. This may be explained by their use of short nucleotides, which differ from those targeted by Folmer primers in this work (651 bp). They also used small population samples in their analysis, which resulted in low intraspecific variation.

In this study, the intraspecific variation in Indonesian palm weevils' genetic diversity was higher than the interspecific variation. Thus, Folmer primers exhibited weak species separation. This result was in contrast to the findings reported by Rugman-Jones et al. (2013). They concluded that interspecific variation is higher than intraspecific variation on the basis of their analysis of COI sequences. Strong species separation was found, and the Asiatic palm weevil and the red palm weevil are valid separate taxa. They analyzed different color morphs of the Asiatic palm weevil via barcoding analysis but did not state that the Asiatic palm weevils have spotted color morphs. Evidence of the color morphs of the Asiatic palm weevil was strengthened by the work of Sukirno et al. (2015), who investigated morphometric variation and interbreeding.

The use of the COI marker for accurate species-level identification requires a high level of interspecific variation that is at least tenfold higher than intraspecific variation (Hebert et al. 2003; Gao et al. 2010). Folmer primers amplified partial sequences of the COI gene (657 bp, 1490–2198 nt) and exhibited 5 highly conserved regions in *R. vulneratus* (the Asiatic palm weevil with rusty red morphs, the Asiatic palm weevil with red stripe, and the Asiatic palm weevil with intermediate color between rusty red and red stripe morphs), *R. bilineatus* (the black palm weevil), and *R. ferrugineus*. The genetic variation within these species was higher than the interspecific variation. This high intraspecific diversity in the COI marker made it unsuitable to distinguish the species (Fig. 1). The difference between the study of Rugman-Jones et al. (2013) and this work suggests that the DNA barcode using Folmer primers provided enhanced sensitivity to genetic diversity (Meusnier et al. 2008). Nevertheless, for species identification, the use of other primers is suggested. This phenomenon of low accuracy of the COI gene in species identification has been observed in other insects, such as blue lycaenid butterflies (Lepidoptera: Lycaenidae) (Wiemers & Friedler 2007) and Diptera (Meier et al. 2006).

The results support previous morphology-based identification, thereby confirming that 2 palm weevil species exist in Indonesia: *R. vulneratus* and *R. bilineatus*. Our findings also confirmed the presence of undescribed spotted rusty red morphs of the Asiatic palm weevil. Thus, the Asiatic palm weevils in Indonesia exhibit a wide range of color polymorphisms, namely, the Asiatic palm weevil with red stripe, the Asiatic palm weevil with rusty red, and the Asiatic palm weevil with intermediate color between red stripe and rusty red morphs.

We observed a high COI diversity in Indonesian *R. vulneratus* and *R. bilineatus* populations, as well as in *R. ferrugineus* from Saudi Arabia. However, the feasibility of using Folmer primers for species identification of these taxa was low. We determined that the Asiatic palm weevil has spotted rusty red polymorphisms, and this species previously was erroneously recognized as the red palm weevil. This determination is consistent with the previous conclusion that there are only 2 palm weevil species, *R. vulneratus* and *R. bilineatus*, in Indonesia.

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