

Proof on the Divergence Times of Two Sympatric Species, *Rhynchophorus ferrugineus* and *R. vulneratus* (Coleoptera: Curculionidae) by Molecular Clock Analysis

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ABSTRACT

Molecular clock analysis on the oil palm weevil has separated the Red Palm Weevil, *Rhynchophorus ferrugineus* and Asiatic palm weevil, *Rhynchophorus vulneratus* as two sympatric species. Calibration was performed using the fossil of *R. cruentatus* which had evolved approximately 1 mya using sequences data of combined *COI* and *Cytb*. Divergence time indicated that *R. cruentatus*, the outgroup species evolved around 80.598±10 mya and finally speciated to form *R. ferrugineus* (≈1.926±10 mya) and *R. vulneratus* (≈4.857±10 mya). Additionally, the construction of Neighbour Joining (NJ) and Maximum Parsimony (MP) trees showed distinct separation using *COI*, *Cytb* and combination of *COI* and *Cytb*. These findings were highly supported by genetic distance analysis. Although a limited number of individuals from the small geographical area was used in this study, the genetic molecular clock analysis of mitochondrial data was able to effectively differentiate both species. These results reveal the first analysis to use the molecular clock to confirm the separation of these two sympatric species.

Key words: Red Palm Weevil, fossil, time divergence, molecular clock, evolution, mitochondria, *COI* and *Cytb*.

INTRODUCTION

Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Olivier, 1970) is categorised under the family Curculionidae of the weevil species and poses a serious threat to the trees of Arecaceae family (Molet, Roda, & Jackson, 2011). The invasion of the RPW significantly reduces the yield of coconut trees in the west coast of Peninsular Malaysia (Idris et al, 2014), and other palm trees e.g. dates and coconut around the world (Mazza et al, 2014). This species has complicated biology, for instance, the pest inhabits inside the tree trunk (Wattanapongsiri, 1966), and the larval and adult stages feed on different resources that renders developing control strategies difficult.

The adult of RPW displays a high degree of color polymorphism, especially for the two species namely *R. ferrugineus* and *R. vulneratus*. This color polymorphism has been discussed and studied by the taxonomists and other researchers. Hallet et al (1993) have studied the aggregation pheromones *R. ferrugineus* and *R. vulneratus* and stated that there was no significant difference between the two species in pheromone production, according to the chemical composition. According to Hallet, Crespi, & Borden (2004), the two colour-morphs in the weevil species, which is having orange or black marking, and black or red stripe are recognized as a single species, *R. ferrugineus*. Hallet et al (2004) have synonymised both species based on the morphological characters, molecular-genetic and breeding data. All that findings have claimed synonymous in both species, consequently invited a great debate among the researchers to prove that both species are cryptic species due to sympatric speciation.

The *R. ferrugineus* and probably its synonymized species, *R. vulneratus* are native from South-East Asia. They have expanded their distributional ranges, which indicates a geographic overlap in both species (*R. ferrugineus* and *R. vulneratus*) (Giblin-Davis, Faleiro, Jacas, Peña, & Vidyasagar, 2013). Rugman-Jones et al (2013) noted that distribution in the *R. ferrugineus* was distributed worldwide and had been found native to the continental southeast Asia (northern and western parts), Sri Lanka and Philippines, while *R. vulneratus* was distributed more to the southern part across Indonesia and had invaded California, U.S.A. It was also suggested that a few cryptic species of the *Rhynchophorus* might exist within the studied populations of both species. Thus, it is permissible to state that both species gathered in this study were species are native to Malaysia and other countries in the native range.

Both species have spread to other countries and regions through the import and export of dates and coconut plants within and outside the country (El-Mergawy et al, 2011). The study by Rugman-Jones et al (2013) has proven that *R. ferrugineus* and *R. vulneratus* are two different species using molecular-genetic of the *COI* mitochondrial data. The phylogenetic tree from the study has also been generated in three distinct lineages of *Rhynchophorus*, which are *R. ferrugineus*, *R. vulneratus* and *R. bilineatus*. The need for thorough research on these two sympatric species was raised and highlighted due to their high degree of polymorphism in coloration (Hallett et al 2004). Due to all the above reasons, both species were claimed as sympatric species, whereby either *R. vulneratus* or *R. ferrugineus* had evolved earlier, but both had finally been found inhabiting the same locality.

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Therefore, this study aimed to investigate the difference between the Red Palm Weevil, *Rhynchophorus ferrugineus* (Olivier) and Asiatic Palm Weevil, *R. vulneratus* (Panzer) (Coleoptera: Curculionidae) using molecular data (*COI* and *Cytb*) by implementing molecular clock analysis to estimate the divergence time of each species. With that, the species separation can be seen clearly.

MATERIAL AND METHODS

Insect sampling and morphological identification

The sampling of palm weevils was conducted between April to June 2013. A total of eight palm weevils were collected from Kuala Selangor, Selangor (*R. vulneratus*) and Kuala Terengganu, Terengganu (*R. ferrugineus*), located at the center and east-coast part of Peninsular Malaysia (Fig. 1). The specimens collected were preserved in 70% alcohol and brought to the Entomology Laboratory in Universiti Kebangsaan Malaysia (UKM) for morphological identification prior to the molecular work. The morphological identification was based on taxonomical keys by Wattanapongsiri (1966) using the StemiD4 stereo microscope.

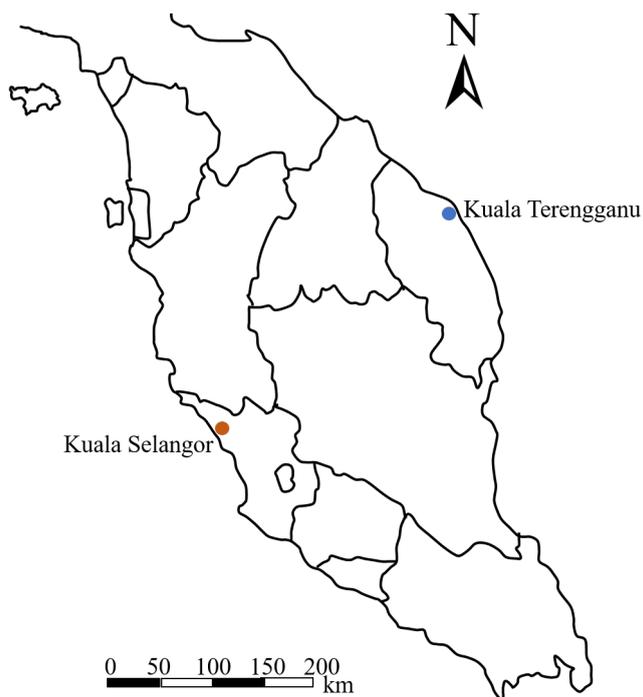


Fig. 1. Map of samples location collected in this study.

DNA extraction and PCR amplification

Tissue samples were taken from the posterior part of the metasomal tergite of the black or red stripe weevil (*R. vulneratus*), and the orange or black marking weevil (*R. ferrugineus*) (Rugman-Jones et al, 2013). DNA extraction of the tissue samples was performed using DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, USA).

The extracted samples were then subjected to PCR amplification. Optimization of PCR amplification for cytochrome oxidase subunit I (*COI*) and cytochrome B (*Cytb*) region were done for each of the individuals. A total of 25 μ l PCR mixture consisted of 0.5 μ l of 0.2mM dNTPs, 10 pmol of each primer, 1.25U of Taq polymerase, and 1 μ l of 15mM MgCl₂ from Vivantis were prepared. PCR was performed using MyGene MG96G Thermalcycler or Thermocycler Perkin Elmer 240 under different conditions for each primer combination, starting with initial denaturation for 3 minutes at 94°C, followed by 39 cycles; denaturation for 1 min at 92°C, annealing for 1 min at 47°C, an extension for 1 min at 72°C and final extension for 5 mins at 72°C (Mohammed, Aman-Zuki, Yusof, S., Md-Zain, & Yaakop 2017; Halim et al, 2018; Aman-Zuki, Mohammed, Md Zain, & Yaakop 2019). Two sets of universal primers used were *COI* [Ron (5' GGA TCA CCT CAT ATA GCA TTC CC 3') (Forward); Nancy (5' CCC GGT AAA AAT TAA AAT ATA AAC TTC 3') (Reverse)] (Simon et al, 1994; Monteiro & Pierce, 2001) and *Cytb* [(CB-J-10933 5' TCT TTT TGA GGA GCW ACW GTW ATT AC 3'; CB-N-11367 5' AAT TGA ACG TAA AAT WGT RTA AGC AA 3')] (Smith, Kambhampati, Völkl, & Mackauer, 1999; Smith & Kambhampati, 1999) to yield 600 bp and 560 bp fragments.

Sequencing and phylogenetic analyses

PCR products for each species were then sent to First Base Sdn. Bhd., Selangor, Malaysia for sequencing. The sequences were then edited using Sequencher 4.8 and aligned using MacClade 4.08. Before the phylogenetic analyses, the genetic distance between the species was obtained for both markers. For phylogenetic analyses (Table 1), the Neighbor Joining (NJ) tree was constructed using Kimura-2 parameter and the bootstrap was analysed (Kimura & Ohta, 1972). The maximum parsimony (MP) tree(s) were generated using PAUP* 4.0- test version 4.0d63 (Swofford, 1998) to get the most parsimonious tree(s). A heuristic parsimony search (Hillis, Moritz, & Mable, 1996) was performed using 100 replicates of random addition sequences, including the TBR (tree bisection reconnection) option for branch swapping. Each base was treated as an unordered character with equal weight, with gaps treated as missing data. Statistical support was obtained by bootstrap analysis with 100 replications (Felsenstein, 1985).

For Bayesian analysis (BI), nucleotide substitution model was selected using jModelTest 2.1.4 (Ronquist et al, 2012). Bayesian Inference trees for *COI*, *Cytb* and combined *COI* and *Cytb* were generated using MrBayes 3.1.2 software with Markov Chain Monte Carlo algorithm (Huelsenbeck, Larget, & Alfaro 2004). The length of the generation chain was analyzed until the value of split frequency is lower than 0.01. The burnin of the final tree was set up at 25% (Drummond, Ho, Phillips, & Rambaut 2006).

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Table 1. List of samples used in the molecular and phylogenetic analyses.

Code sample	Species	Locality	Accession No. <i>COI</i>	Accession No. <i>Cytb</i>
K01	<i>Rhynchophorus vulneratus</i>	Malaysia: Selangor, Kuala Selangor	MG051024	MG051032
K02	<i>Rhynchophorus vulneratus</i>	Malaysia: Selangor, Kuala Selangor	MG051025	MG051033
K04	<i>Rhynchophorus vulneratus</i>	Malaysia: Selangor, Kuala Selangor	MG051026	MG051034
K05	<i>Rhynchophorus ferrugineus</i>	Malaysia: Terengganu: Kuala Terengganu	MG051027	MG051035
K06	<i>Rhynchophorus ferrugineus</i>	Malaysia: Terengganu: Kuala Terengganu	MG051028	MG051036
K07	<i>Rhynchophorus ferrugineus</i>	Malaysia: Terengganu: Kuala Terengganu	MG051029	MG051037
K08	<i>Rhynchophorus ferrugineus</i>	Malaysia: Terengganu: Kuala Terengganu	MG051030	MG051038
K10	<i>Rhynchophorus ferrugineus</i>	Malaysia: Terengganu: Kuala Terengganu	MG051031	MG051039
GBMIN32436	<i>Rhynchophorus ferrugineus</i>	Japan	GU581524	-
GBMIN32437	<i>Rhynchophorus ferrugineus</i>	Japan	GU581522	-
GBMIN32267	<i>Rhynchophorus ferrugineus</i>	Japan	GU581521	-
GBMIN32268	<i>Rhynchophorus ferrugineus</i>	Japan	GU581519	-
GBMIN32269	<i>Rhynchophorus ferrugineus</i>	Japan	GU581518	-
GBMIN32439	<i>Rhynchophorus ferrugineus</i>	Japan	GU581517	-
	<i>Rhynchophorus palmarum</i>	-	GU581629	GU581656
	<i>Rhynchophorus cruentatus</i>	-	AY131113	

Molecular clock analysis

The analysis to measure and estimate the time divergence of the RPW species was done using a combination sequence data of *COI* and *Cytb*. Two compatible softwares used for the time divergence analysis were BEAUti and BEAST version v1.10.4 (Suchard et al, 2018). The nucleotide substitution model was chosen using jModelTest (Ronquist et al, 2012) prior to analysis. The chosen model was HKY+G. The clock partitioning was set for ingroup and outgroup taxa with *R. cruentatus* (AY131113) as outgroup and set with tip date from time fossil for Palmetto Weevil, *Rhynchophorus cruentatus* Fabricius (Insecta: Coleoptera: Curculionidae) which is 1 million years ago (Weissling & Giblin-Davis, 1997). Lognormal relaxed clock was chosen for clocks model referring to Heath, Huelsenbeck, & Stadler (2014) to remove the assumption of a strict molecular clock in the uncertain or unresolved topology of phylogenetic tree. The Speciation: Birth-Death Process was chosen for the tree before estimation of the speciation with the rate of birth (branching speciation) and death (extinction) incorporated in the lineage following (Stadler, Gavryushkina, Warnock, Drummond, & Heath, 2018) and (Herbst, 1795). The time fossil for *Rhynchophorus* used was $48.6-40.4 \pm 10$ mya as reported from (Rambaut & Drummond, 2015). The length of the chain for this analysis was fixed at 10,000,000 generations of Markov Chain Monte Carlo with tree sampling at every 1000 generations. The burnin of the final tree was set up at 25% from total tree using TreeAnnotator 1.7.5 (Drummond & Rambaut, 2007). Molecular clock tree was visualized using FigTree 1.4.2 (Posada, 2008).

RESULTS

Morphological identification

The weevil samples were identified based on the color morph according to Wattanapongsiri (1966) description, in which orange with black marking was characterized for the *R. ferrugineus*, while black with red stripe was for the *R. vulneratus*. Three individuals collected from Kuala Selangor were identified as *R. vulneratus* (K1-K2, K4), while other specimens collected from Kuala Terengganu (K5-K8, K10) were identified as *R. ferrugineus*.

Phylogeny tree reconstruction and genetic distances

All individuals were successfully extracted and amplified using both markers, viz. *COI* and *Cytb* in the length of 600 and 560 bp. Phylogeny analyses have confirmed that both spotted and striped weevils were located in different clades for each molecular data in NJ, MP and BI analyses (Figs. 2a-c, 3a-c and 4a-c). In the phylogeny results, *Rhynchophorus vulneratus* and *R. ferrugineus* are located in different clades and supported with 100% bootstrap values in both analyses. The specimens from Japan were added in the *COI* datasets and yet still showed the separation of the outgroup and ingroups (*R. cruentatus* and *R. palmarum*) at different lineages and highly supported with 100% bootstrap value. The genetic data differences showed that individuals of the *R. ferrugineus* from Malaysia and Japan were 0.047 (Table 2).

Table 2 and 3 show the genetic distance for individuals in *R. vulneratus* and *R. ferrugineus* using *COI* and *Cytb* data. Genetic distance for both *COI* and *Cytb* markers showed that individuals among the *R. vulneratus* had high divergences compared to *R. ferrugineus*. In *COI*, genetic distances among the *R. vulneratus* were 0.010-0.029, while among *R. ferrugineus* the values were 0.000-0.047. In *Cytb*, genetic distances among *R. vulneratus* were 0.016-0.019, while among *R. ferrugineus* the values were 0.000-0.05.

Estimating divergence time

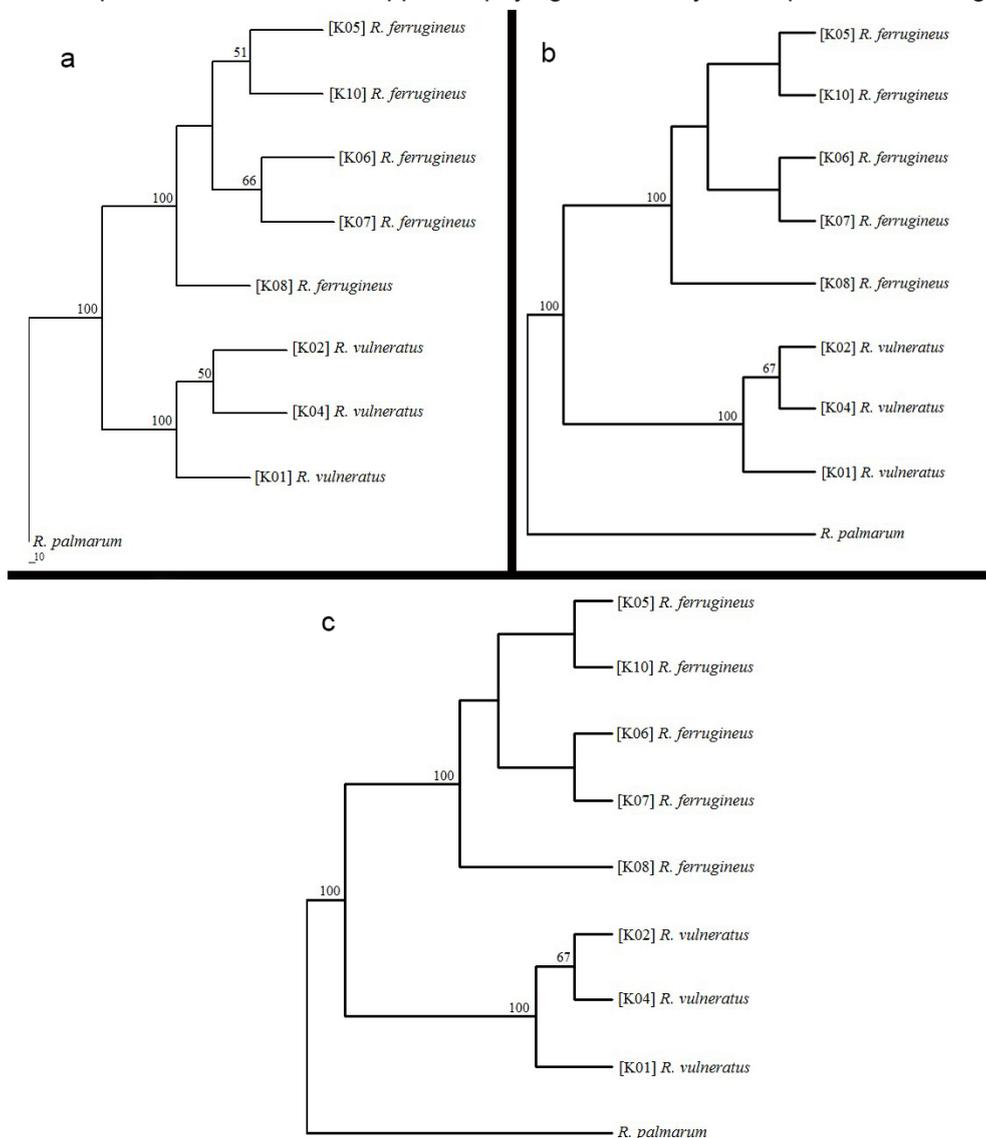
The result of molecular clock analysis (Fig. 5) based on the combination of *COI* and *Cytb* (1367 bp) molecular data showed different divergence times in both sympatric species (*R. vulneratus* and *R. ferrugineus*) and the outgroup species, *R. cruentatus* Fabricius (Coleoptera: Curculionidae). The divergence times showed that *R. cruentatus* had diverged earlier compared to *R. vulneratus* and *R. ferrugineus* since around 80.598 ± 10 mya followed by *R. vulneratus* at $\approx 4.857 \pm 10$ mya and the latest was *R. ferrugineus* at $\approx 1.926 \pm 10$ mya.

DISCUSSION

In this paper, the Malaysian samples were taken as a model species to measure the time divergence of *Rhynchophorus ferrugineus* and *R. vulneratus* from the Oriental region. Although a small-scale area (Peninsular Malaysia only) was considered in

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this study, it was very significant in influencing the speciation process. In this study, *R. palmarum* was selected as the outgroup for comparing the two cryptic species. According to Löhr, Vásquez-Ordóñez, & Lopez-Lavalle (2015), the cryptic species (*R. ferrugineus* and *R. vulneratus*) was distinctly separated from the *R. palmarum* using *COI* sequences data with the support of phylogenetic analysis in species clustering.

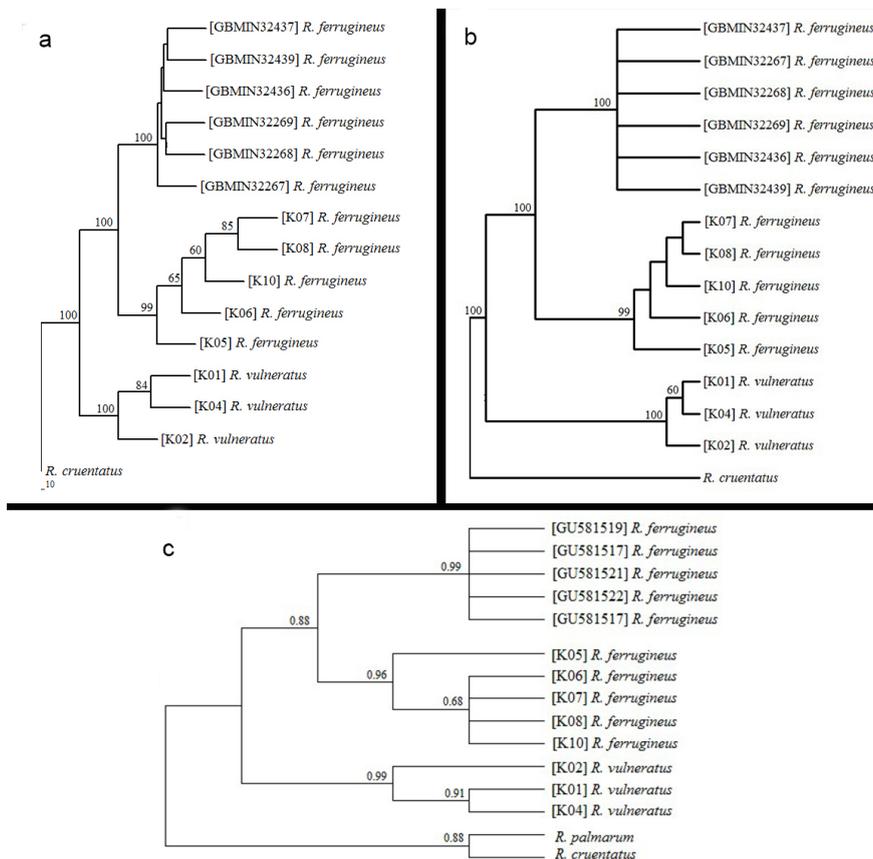


Figs. 2a-c. Phylogenetic tree of the *Rhynchophorus ferrugineus* and *R. vulneratus* based on NJ (a), MP (b) and BI (c) analyses using *Cytb* data sequences.

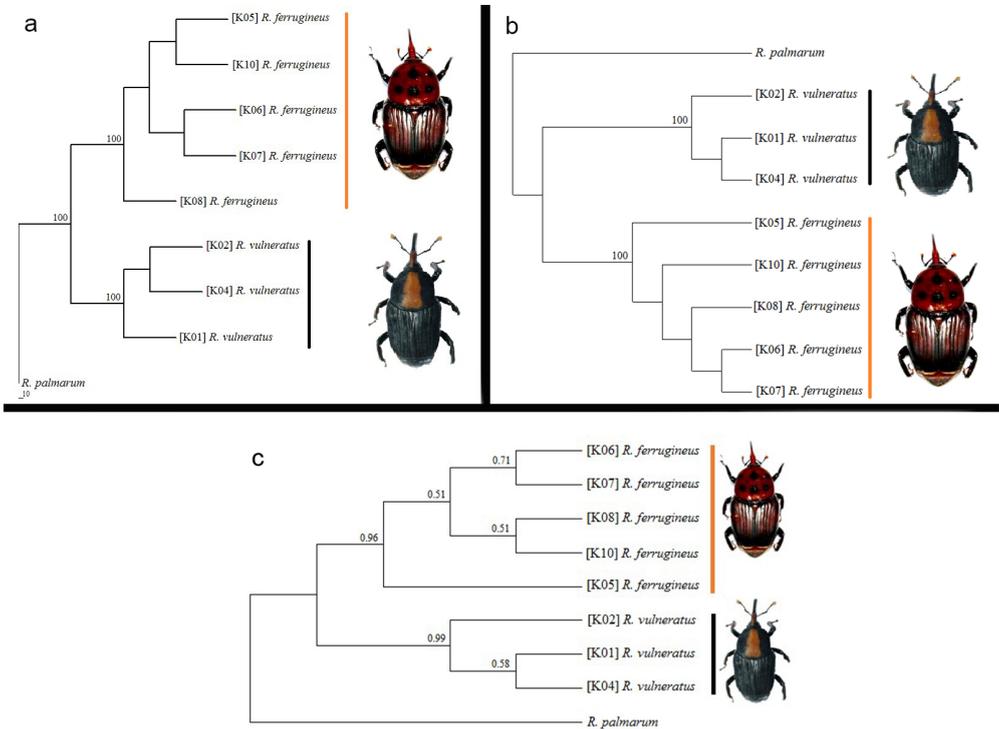
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Table 3. Genetic distance of species, *R. vulneratus*, *R. ferrugineus* and *R. palmarum* using *Cytb* data.

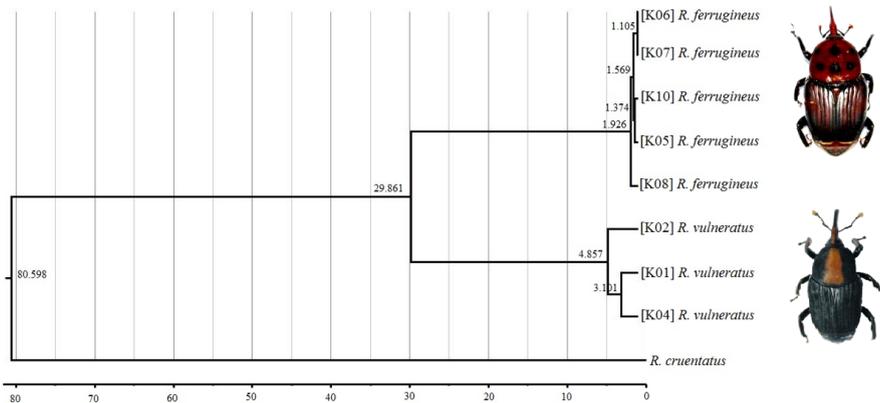
	K01	K02	K04	K05	K06	K07	K08	K10	<i>R. palmarum</i>
[K01] <i>R. vulneratus</i>	-								
[K02] <i>R. vulneratus</i>	0.019								
[K04] <i>R. vulneratus</i>	0.019	0.016							
[K05] <i>R. ferrugineus</i>	0.146	0.154	0.149						
[K06] <i>R. ferrugineus</i>	0.143	0.157	0.153	0.003					
[K07] <i>R. ferrugineus</i>	0.143	0.157	0.153	0.003	0.000				
[K08] <i>R. ferrugineus</i>	0.150	0.157	0.153	0.003	0.005	0.005			
[K10] <i>R. ferrugineus</i>	0.150	0.157	0.153	0.003	0.005	0.005	0.005		
<i>R. palmarum</i>	0.282	0.292	0.282	0.294	0.290	0.290	0.299	0.300	-



Figs. 3a-c. Phylogenetic tree of the *Rhynchophorus ferrugineus* and *R. vulneratus* based on NJ (a), MP (b) and BI (c) analyses using *COI* sequences data.



Figs. 4a-c. Phylogenetic tree of the *Rhynchophorus ferrugineus* and *R. vulneratus* based on NJ (a), MP (b) and BI (c) analyses using combination of *Cytb*+*COI* sequences data.



Figs. 5. Molecular clock tree of *Rhynchophorus ferrugineus* and *R. vulneratus* using combination of *Cytb* + *COI* sequences data.

Even though the body colour and spots could not clearly differentiate the two studied species, *R. ferrugineus* and *R. vulneratus*, the *COI* marker on the other hand,

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has shown a species separation. Although a small number of weevil specimens was examined in this study as compared to that of (Rugman-Jones et al 2013), a study of the species time divergence to clarify the *R. ferrugineus* and *R. vulneratus* as a sympatric species would be the most effective method and a significant item to be measured and investigated, despite the fact the factors for the speciation of the species are still very much in doubt. In addition, only a small scale of samples need to be utilized to elucidate a complex morphology or cryptic species. Likewise, there had also been similar studies on the different genetic data among cryptic species of gecko (Oliver, Adams, & Doughty, 2010), and we believed that this could reveal a similar pattern in insect species.

Previous studies on *R. ferrugineus* and *R. vulneratus* only focused on either morphological (Wattanapongsiri, 1966; Sazali, Hazmi, Abang, & Jemain, 2018) or molecular (Rugman-Jones et al, 2013) aspects but none reported on the incorporation of both aspects and species, except by Hallet et al (2004). As a result, various conclusions regarding the species status of *R. ferrugineus* and *R. vulneratus* were presented. According to Wattanapongsiri (1966), the identification of *R. ferrugineus* and *R. vulneratus* was mainly based on the colour morph, the shape of the pronotum as the second morphological characters while Rugman-Jones et al (2013) debunked that the pronotum shape was one of the distinguished characters in two species. A study by Sazali et al (2018) reported that the morphological differences between *R. ferrugineus* and *R. vulneratus* were significant in terms of the size of the species. *Rhynchophorus vulneratus* is slightly larger compared to *R. ferrugineus* with the length of pronotum for *R. vulneratus* is measuring 12.87 mm, while for *R. ferrugineus* it is 11.51 mm, and the mean width of elytra is 13.60 mm, it is 11.44 mm for *R. vulneratus* and *R. ferrugineus*, respectively.

Results from the molecular studies indicated that *R. ferrugineus* and *R. vulneratus* differed from each other and this was supported by the morphological features depicted by Wattanapongsiri (1966). According to Hallett et al (2004), the crossbred of *R. ferrugineus* and *R. vulneratus* have successfully produced a fertile F1. However, the survival of the hybrid was not further discussed in the paper. Additionally, they provided a weak single proof of DNA similarity in which only 201 bp of mitochondrial DNA sequence in the *COI* gene were identical between the two species. In addition, a study by Sukirno et al (2018) had confirmed that the undescribed color polymorphism existed in Indonesia *R. vulneratus* samples, in which rusty red polymorphisms had been wrongly identified as *R. ferrugineus*. The low interspecific variability was also detected in *COI* sequences in the Indonesia and Saudi Arabia samples, despite *COI* having proven to be a great marker for species separation. Sukirno, Tufail, Rasool, & Aldawoo (2020) also had stated that pronotal markings and *COI* sequences were able to separate the *R. ferrugineus* under three haplotypes number. Moreover, in this study, the *COI* marker has proven effective in species separation, especially on the Malaysian samples, but using different primers that have not been used earlier by Sukirno et al (2018) and Sukirno, Tufail, Rasool, Husain, & Aldawood (2020b).

Referring to Filchak, Roethele, & Feder (2000), speciation may occur due to changes in hosts and does not only depend upon geographical isolation integrating sterility and life development. We assumed that the changing of the pronotum coloration in both *Rhynchophorus* species from orange or black marking (*R. ferrugineus*) to black or red stripe (*R. vulneratus*) or vice versa indicated major changes in the evolution and speciation to finally become two distinct species. A study by Kelley, Fitzpatrick, & Merilaita (2013) also reported that the coloration and spots of coral reef fishes were investigated, and they found that the presence of spots and stripes on the fish body were correlated with several parameters. Due to this, fish biology seems to be developing as a whole in parallel with the fish's evolution, for example in body length, feeding behavior and habitat preferences.

To date, no clear studies have been performed on biological and geographical factors that separate the weevil species from Malaysia and other areas in their native range. Furthermore, the divergence time for both species took place much earlier at $\approx 29.861 \pm 10$ mya. The molecular clock analysis also revealed that *R. vulneratus* formed as a new species earlier at $\approx 4.857 \pm 10$ mya as compared to *R. ferrugineus* at $\approx 1.926 \pm 10$ mya. These findings provide a baseline data of molecular clock study in *Rhynchophorus* spp. regardless of the sample size.

In terms of evolutionary processes, we hypothesize that the changes in coloration from a fully black weevil (*R. palmarum*) to black or red stripe (*R. vulneratus*) is a characteristic that commonly changes through evolution. For example, a similar change took place in the *Timema* species of walking sticks in their dorsal stripes through their interactions with the host plants (Sandoval & Crespi, 2008; Farkas et al, 2013). However, this process required a very long time due to geographical isolation in parallel with a gradual process of speciation that led to reproductive isolation (Mayr, 1942). In this situation, we believed that *R. palmarum*, which is native to South America and countries nearby, took approximately 500,000 years to become *R. vulneratus* is native to Southeast Asia and Indo-Malaya.

Furthermore, both sympatric species started diverging into different subpopulations from 29.861 mya and started to speciate after a relatively short time, approximately 3 mya. This may have been influenced by changing the host plant from the coconut tree to the date tree. We believed that the process started in an area where both coconut and date trees grew together. For this reason, competition occurred that correlated with the availability of the plant host itself as a food source. A population of *R. vulneratus* changed its host plant from coconut to date trees and had speciated to form *R. ferrugineus* within a short time. Similar events have happened in species not limited to insects as seen in studies by Bush et al (1989), Bush & Butlin, (2004), but also in other kinds of animal species, for example in the coral-dwelling fish genus *Gobiodon* (Munday, Van Herwerden, & Dudgeon, 2004).

The results of the molecular clock analysis of this study supported that *R. ferrugineus* and *R. vulneratus* were two valid species with *R. vulneratus* being formed as a new species earlier than *R. ferrugineus*. The time of separation for *R. ferrugineus* and *R. vulneratus* is generally accepted as that for the separation of *R. ferrugineus*,

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which for *R. bilineatus*, *R. phoenicis*, *R. cruentatus* and *R. palmarum* was from 2.3 to 3.7 million years ago. Furthermore, a study by Hallett et al (2004) stated that *R. ferrugineus* and *R. vulneratus* had separated from *R. bilineatus* about 5 million years ago.

In this study, the genetic distance among members of *R. vulneratus* was higher than that of *R. ferrugineus* for both markers, indicating a high divergence in *R. vulneratus* species (Figs. 2-4, Table 2-3). However, the value of the *COI* obtained from the genetic distance analysis was lower (0.000-0.047) compared to the values obtained by El-Mergawy et al (2011) in *R. ferrugineus* at a range of 0.058–0.095. Even though the results showed slight difference in the genetic distance value however, there are a few assumptions that can be made regarding this situation. Firstly, there may be cryptic species within *R. vulneratus* that have created a higher genetic distance value (Rugman-Jones et al, 2013). Secondly, the occurrence of sympatry within the *R. vulneratus* species may create higher values for genetic distance. Sympatry within species has been reported in Curculionidae, for example in the *Laparocerus* weevil (Faria et al, 2016) and *Mecinus* weevil (Toševski et al, 2011). Furthermore, high genetic diversity is a characteristic exhibited in the native range of the species. For example, the high genetic diversity in *R. ferrugineus* from Pakistan indicates that this species is native to Pakistan (Yasin, Rugman-Jones, Wakil, & Stouthamer, 2016). Molecular clock studies provide important information regarding the species being studied. In our study, molecular clock analysis was able to prove the status of *R. ferrugineus* and *R. vulneratus* as two valid species. Likewise, a study by (Zhang et al, 2005) provided proof of the existence of a *Coptalabrus* species (Carabidae) using the divergence time derived from a phylogeographic study of this species in South Korea. This demonstrates that information regarding divergence time is important for phylogeographic study. This is because phylogeographic study involves the evolutionary history, population genetics, geography and divergence time of the species (Edwards & Beerli, 2000).

CONCLUSION

Molecular clock analysis using the combined data of *COI* and *Cytb* has firmly separated the sympatric species, *R. ferrugineus* and *R. vulneratus*. Based on the divergence time, we have discovered that the species speciate to form *R. ferrugineus* from $\approx 1.926 \pm 10$ mya and *R. vulneratus* $\approx 4.857 \pm 10$ mya. The separation was also supported by phylogenetic analyses of NJ, MP and BI, as well as proven by the data of the distance analysis. The information gathered by this study will be able to provide information for future regional studies related to the RPW.

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