# GENETIC VARIATION AND INVASION HISTORY OF THE INVASIVE RED PALM WEEVIL (*Rhynchophorus ferrugineus* (OLIVIER)) IN TERENGGANU

Chong J. L.
*Kenyir Research Insitute & School of Marine & Environmental Sciences*
*Universiti Malaysia Terengganu,*
*21030 Kuala Terengganu, Terengganu, Malaysia*
*julian@umt.edu.my*

H'ng, T. M.
*School of Marine & Environmental Sciences*
*Universiti Malaysia Terengganu,*
*21030 Kuala Terengganu, Terengganu, Malaysia*
*h.taimei14@gmail.com*

Wahizatul Afzan Azmi
*School of Marine & Environmental Sciences*
*Universiti Malaysia Terengganu,*
*21030 Kuala Terengganu, Terengganu, Malaysia*
*wahizatul@umt.edu.my*

Noor Hasmiza Amansuria
*School of Marine & Environmental Sciences*
*Universiti Malaysia Terengganu,*
*21030 Kuala Terengganu, Terengganu, Malaysia*
*osakurataxo@yahoo.com*

**ABSTRACT**

*In 2007, the red palm weevil (RPW) Rhynchophorus ferrugineus, was first detected on coconut palms by the Terengganu Department of Agriculture (DOA) in Terengganu districts. As of current time, it has been recorded in other states including Kelantan, and Pahang. As a lethal pest of coconut, sago and oil palm, early symptoms of R. ferrugineus are hard to detect, with detection of its final stages only when the tree is beyond saving. Well- known as an invasive species, knowledge of the genetic make up of R. ferrugineus is important, as it will contribute valuable information to a more detailed program to monitor and control it before it adapts to one of Malaysia's cash crop, the oil palm. As R. ferrugineus is similar in morphology to another native species R. vulneratus, a molecular marker namely cytochrome c oxidase subunit 1 (CO1) gene was utilised to verify the identity of suspected R. ferrugineus samples and to compare genetic variations within different morphs of R. ferrugineus in Terengganu. For this study, 30 individuals of R. ferrugineus were chosen, with 5 samples each from six Terengganu districts. A 600 bp product was obtained from the Polymerase Chain Reaction (PCR) conducted which was then sequenced. Results revealed that all 30 samples were 100% similar in nucleotide bases and is same with the R. ferrugineus DNA sequence in the National Centre for Biotechnology Information (NCBI) database, which verifies its presence and existence in Malaysia. Additionally, the haplotype obtained in this study was the H8 haplotype, previously recorded from the Mediterranean area. High genetic similarity between samples suggest that the Terengganu populations were from a single founder population, which is common in invasive species. Findings of the haplotype H8 is concurrent with current hypothesis of the R. ferrugineus origins from Middle East. Recent studies that the newly discovered R. ferrugineus at Fujian, China shares the same haplotype with those in Terengganu. More studies are recommended in order to investigate other possible invasive pathways of R. ferrugineus.*
*Keywords: Red palm weevil, Rhynchophorus ferrugineus, genetic variation, mitochondrial cytochrome oxidase 1 (CO1).*

Key words: Unemployment, Unemployment Insurance, Social Insurance, Social Security, Iran Unemployment Insurance Law.

## Introduction

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) is reported as a lethal pest of coconut, sago and oil palm (Malumphy & Moran, 2009), and mostly attack young trees that are less than 20 years old (Abraham *et al.*, 1998; Faleiro, 2006). Previously, it was recorded as a minor pest of coconut trees in Malaysia and Thailand (Murphy & Briscoe, 1999). In 2007, a serious infestation of this pest was first detected by the Malaysian Department of Agriculture (DOA) in 58 locations in all the seven Terengganu districts where an intensive three-month survey in 2011 throughout Terengganu in over 800 ha of coconut plantations, parks, villages and in the Federal Land Development Authority (FELDA) plantations revealed that *R. ferrugineus* attacked some 550,000 coconut trees in Terengganu indicating a dramatic increase and speedy distribution of this pest (Wahizatul *et al.*, 2013).

Early symptoms of *R. ferrugineus* infestation are difficult to be detected (EPPO, 2008), as it spends its early life stages within the tree trunk. Then, it will destroy the vascular system and bore into the heart of the host plant which may lead to tree collapse (Ju *et al.*, 2011). Therefore, only the final stage of symptoms such as fallen empty pupal cases (Faleiro, 2006) will be detected.

Being an invasive species, *R. ferrugineus* is able to replicate and spread very fast as they only need a short period of about 3 to 4 months to complete their life cycle (Hussain *et al.*, 2013). The history of *R. ferrugineus* invasion and the relationships between its different geographic populations should be understood in order to have a better management plan to deal with it. As such, knowledge of genetic variations in *R. ferrugineus* as an invasive species is essential in order to know the speedy adaptation of this lethal pest in coconut trees. Besides that, studies on the genetic diversity of *R. ferrugineus* will assist in discovering their invasion routes and the number of introduction events that have may occurred in each invaded or colonized countries. Moreover, genetic variation studies among the various geographic populations of invasive species is necessary for designing their management strategy, which includes biosecurity as it will enable speedy and accurate identification of the alien species and an exact characterization of their populations**.**

Molecular markers are key players in animal genetics to show polymorphisms at the DNA level (Vignal *et al.*, 2002). These markers are usually considered to measure neutral DNA variation and hence are beneficial in studies of species relationships (phylogenetic), hybridization, gene flow, fingerprinting and genetic structure of populations and other purposes (USDA, 2006). Besides that, molecular markers can be used to identify the natural history and evolution of certain organisms (Avise, 1994). According to Mostoskey *et* al. (2000), molecular markers can used to identify genes that carry certain diseases, genetic engineering, paternity testing and forensics. Additionally, DNA markers are effective tools in making inferences about movement of insect populations, because they represent selectively neutral characters (Black *el al.*, 2001).

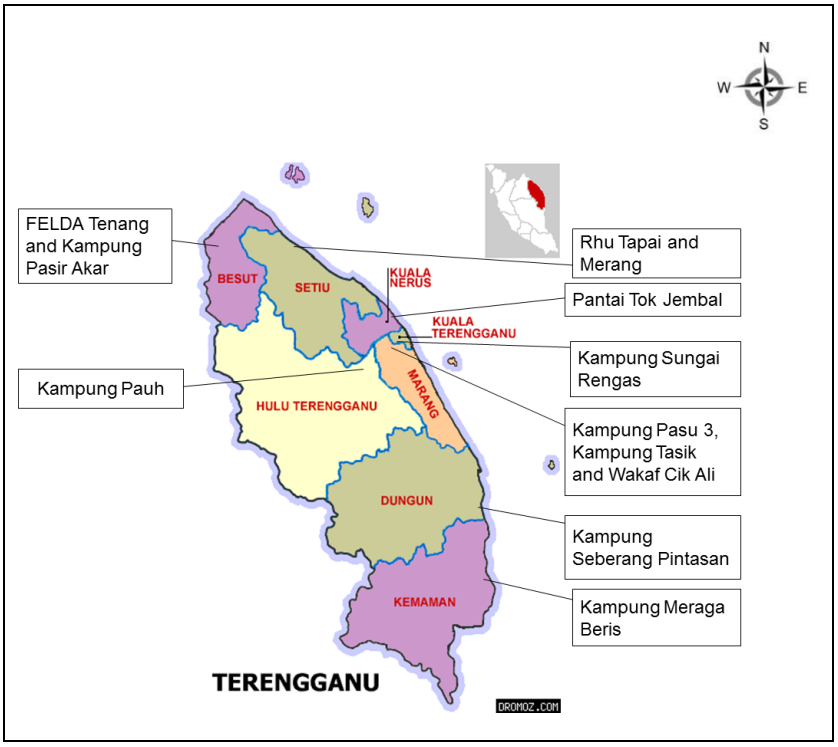
Information on the genetic diversity of *R. ferrugineus* in Terengganu and Malaysia is quite limited because there are not much studies conducted on the genetic aspect of this pest previously. As such, this study aims to identify and to differentiate *R. ferrugineus* from other *Rhynchophorus* species using the mitochondrial cytochrome c subunit 1 (CO1) marker. CO1 was used to investigate the origins and invasion history of *R. ferrugineus* as it had been proven effective to study genetic variation in *R. ferrugineus* (El-Mergawy *et al.*, 2011; Rugman-Jones *et al.*, 2013; Wang *et al.*, 2015). Additionally, as *R. ferrugineus* exhibit various variations in adult sizes, colours, prenatal markings and shape of prenatal markings which sometimes can be confused with *Rhynchophorus schach* Olivier, a serious pest of sago palm and coconut palm in Malaysia which commonly known as Red Stripe Weevil.

**Methodology**

Terengganu was chosen as the focal sampling site as the presence of exotic *R. ferrugineus* was first reported in Terengganu in early 2007. Its presence was reported to cause the death of three common coconut cultivars in 58 Terengganu locations in early 2007, and reaching a peak of 858 localities in Terengganu which reported the presence of *R. ferrugineus* in 2011.

Preliminary location data indicating areas with high density of *R. ferrugineus* provided by Department of Agriculture, Terengganu was used to identify sampling sites. The sampling sites chosen at the 8 districts of Terengganu were FELDA Tenang and Kampung Pasir Akar, Besut; Rhu Tapai and Merang, Setiu; Pantai Tok Jembal, Kuala Nerus; Kampung Sungai Rengas, Kuala Terengganu; Kampung Pasu 3, Kampung Tasik and Wakaf Cik Ali, Marang; Kampung Seberang Pintasan, Dungun; Kampung Pauh, Hulu Terengganu and Kampung Meraga Beris, Kemaman (Figure 1). Pheromone traps consisting of pheromone, 500 to 600 mL water and food bait of 300 to 400 g pineapple or sugarcane were set up to collect the *R. ferrugineus* samples. As there were no *R. ferrugineus* samples collected at Hulu Terengganu and Kemaman districts due to time constraints, a total of 30 samples of *R. ferrugineus* were collected with 5 samples each from six districts.

Figure 1: Eight districts of Terengganu. Source: Dromoz.com




For Deoxyribonucleic Acid (DNA) extraction, all 30 fresh samples of *R. ferrugineus* were immersed into 70% alcohol before separation of the head and body. Fresh tissue (25 mg) were then taken and genomic DNA extracted using DNeasy Tissue Kit (QIAGEN) according to the manufacturer's protocol. DNA concentration of all extracted samples was confirmed by running 1% of agarose gel electrophoresis by comparing with Lambda Hind III marker. The electrophoresis was conducted at 90 volt for 55 minutes in 1x TAE buffer.

Polymerase Chain Reaction (PCR) was performed in a total volume of 25 µL containing 1x reaction buffer, 3 mM $MgCl_2$, 0.24 mM dNTPs, 1.4 µM of each primer [Bron (5'-TATAGCATTCCCCGTTTA-3' and Simon (5'-TCCTAATAAACCAATTGC-3') modified from Simon *et al.* (1994) ], 1 U Go Taq Flexi DNA polymerase (Promega Corp) and 2.5 µL of DNA (a 100 time dilution of the original DNA). The PCR program was as follows: 94°C for 5min, followed by 40 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 1 min, and a final extension at 72 °C for 5 min (El-Mergawy *et al.*, 2011). PCR products were purified using QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's protocol. Purified PCR products were analyzed by electrophoresis on 1% agarose gel in 1x TAE buffer. Electrophoresis was conducted at 90 volts for 55 minutes. The molecular size of the amplified products was estimated using 100bp DNA ladder (Invitrogen) and the bands analyzed using Image Master Documentation System (VDS). Only the forward strand was sequenced.

All the sequences of *R. ferrugineus* were aligned manually by using Multiple Alignment ClustalW© in order to calculate the percentage of similarity and dissimilarly nucleotide bases. Chromas® software was used to remove invalid and unwanted start and end point. In order to perform sequence similarity searches, all sequences were subjected to Basic Local Alignment Search Tool (BLAST). Phylogenetic trees were constructed using two different construction methods: (a) Neighbour joining (NJ) and (b) maximum likelihood (ML). The NJ tree was constructed using Tamura-Nei model, while ML tree was constructed using Kimura-2 parameter model. Genealogical analyses were conducted in MEGA6 software, with bootstrap support values obtained from 1000 replications using both methods. *Rhynchophorus vulneratus* was used as an out-group taxon.

**Results and discussion**

For this study, PCR amplification produced a single amplified DNA band of the CO1 gene of 600bp for 30 *R. ferrugineus* samples which was then sequenced. All sequences were checked using Basic Local Alignment Search Tool (BLAST) in order to perform sequence similarity searches with *R. ferrugineus* (accession number: GU581319.1) and for the out-group, *Rhynchophorus vulneratus* (accession number: KF311627.1). All invalid and unwanted starting point and ending point were eliminated manually and produced final products of only around 500bp in length for *R. ferrugineus*. Next, sequences were aligned by using Sequences Multiple Alignment ClustalW to determine the similarities that exist between samples within each district. A conserved region of about 500bp was chosen, from base 46 to 533 for comparison.

Based on Basic Local Alignment Search Tool (BLAST) results, the results revealed that all the samples obtained in this study are 100% similar to *R. ferrugineus* (accession number: GU581319.1), thus proving that CO1 is an effective molecular marker for the identification of *R. ferrugineus.*

Two types of phylogenetic trees were constructed using different models. The neighbor joining tree (Figure 2) was constructed using Tamura-Nei model while maximum likelihood tree (Figure 3) was constructed using Kimura-2 parameter; boostrap values: 1000. There are 0.00% of differences between all 30 individuals, meaning all of them are categorized under same species, *Rhynchophorus ferrugineus. Rhynchophorus vulneratus* as an out-group taxa showed an 0.07 distance with all samples.

Figure 2: Neighbor joining tree of 30 samples of *Rhynchophorus ferrugineus* from Terengganu districts and *Rhynchophorus vulneratus* used as an out-group.

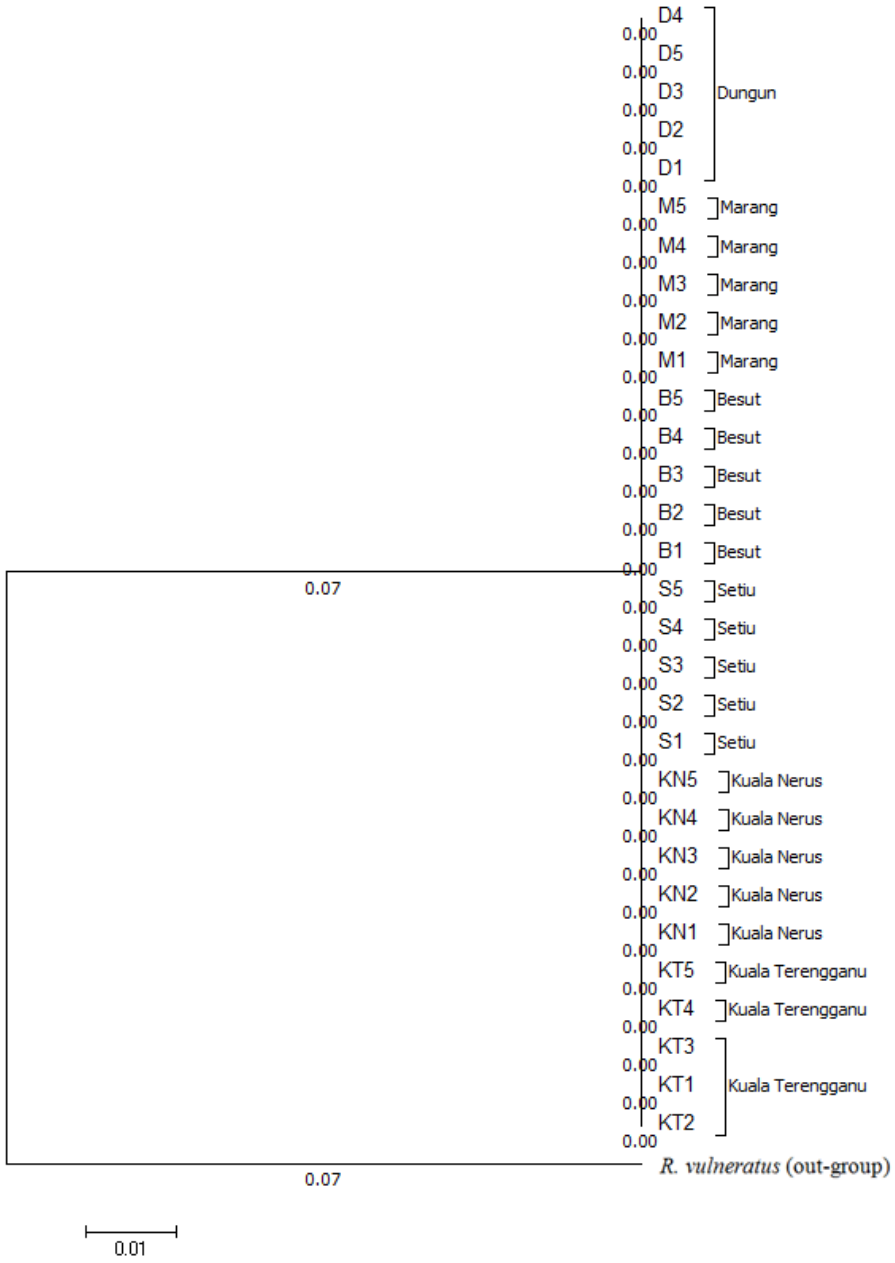
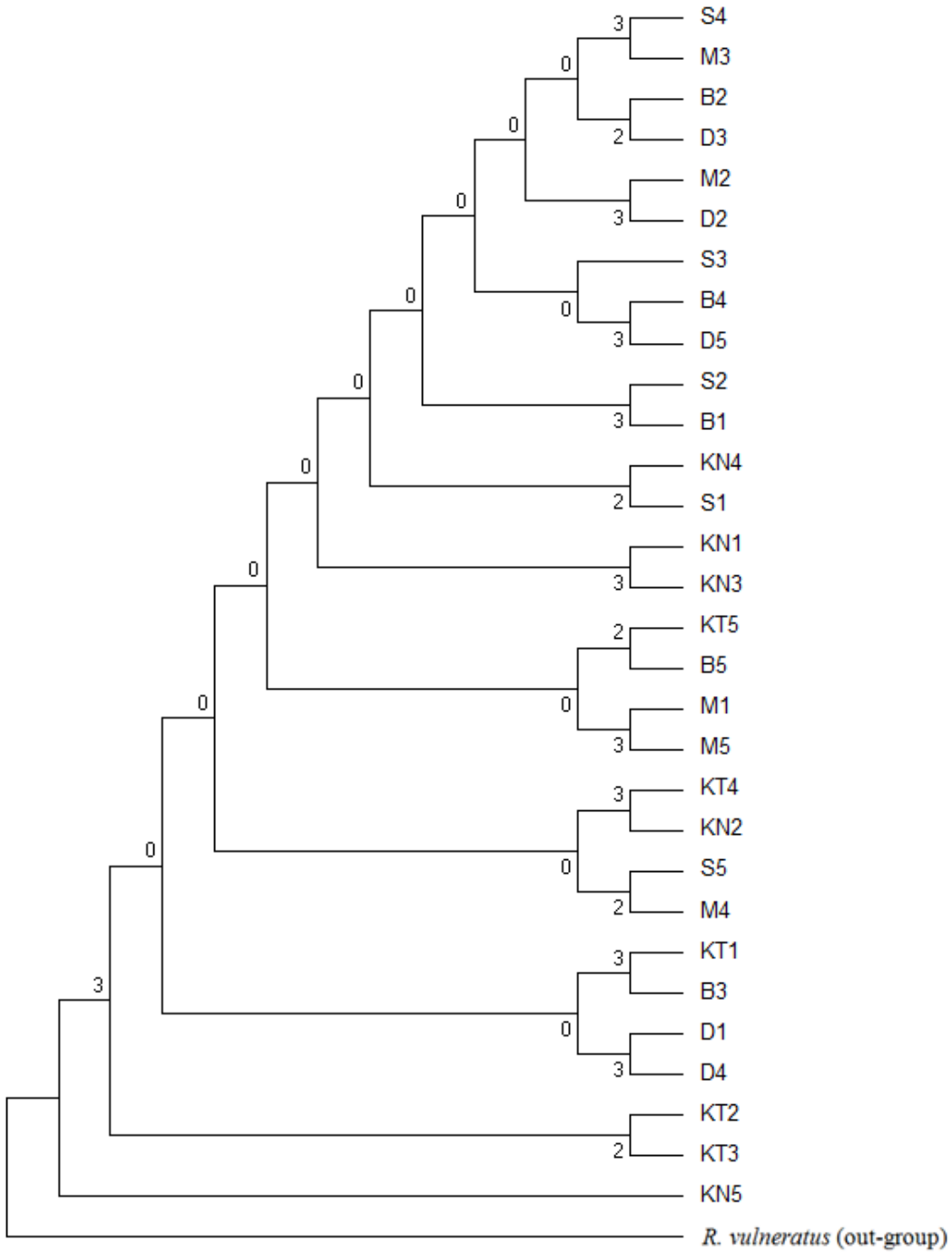

D4
0.00
D5
0.00
D3
0.00
D2
0.00
D1
0.00
Dungun
M5
0.00
M4
0.00
M3
0.00
M2
0.00
M1
0.00
Marang
B5
0.00
B4
0.00
B3
0.00
B2
0.00
B1
0.00
Besut
0.07
S5
0.00
S4
0.00
S3
0.00
S2
0.00
S1
0.00
Setiu
KN5
0.00
KN4
0.00
KN3
0.00
KN2
0.00
KN1
0.00
Kuala Nerus
KT5
0.00
KT4
0.00
KT3
0.00
KT1
0.00
KT2
0.00
Kuala Terengganu
R. vulneratus (out-group)
0.07
0.01

Figure 3: Maximum likelihood tree of 30 samples of *Rhynchophorus ferrugineus* from Terengganu districts and *Rhynchophorus vulneratus* used as an out-group.




The bootstrap values are indicated at above and ranged from 0 to 3.

Among all the 30 samples, no insertion or deletion of nucleotide bases was detected. All samples from different districts showed 100% similarity of nucleotide bases and thus, no variations among the individuals were detected. Previous studies of genetic variation in *R. ferrugineus* specimens utilizing the CO1 gene showed high similarity in *R. ferrugineus* populations (El-Mergawy *et al.*, 2011; Rugman-Jones *et al.*, 2013; Wang *et al.*, 2015). In this study, there were no differences in nucleotide bases between samples from different districts of Terengganu. According to Dlugosch & Parker (2008), all invasive species have experienced population founder events and many of them have decreased genetic variation in their introduced ranges. In this study, no differences in nucleotide bases between samples from different districts was detected which means founder events happened to the original populations of *R. ferrugineus*. This indicates that the *R. ferrugineus* populations in Terengganu originated from a very small number of individuals which established themselves in Terengganu, which in turn contributed to the lack of genetic diversity in *R. ferrugineus* populations in Terengganu.

A single haplotype was obtained in this study and was compared with the previous studies of *R. ferrugineus*. Results showed that the haplotype obtained in this study matched the H8 haplotype (El- Mergawy *et al.*, 2011) (Table 1 and 2). This may be explained by a unique introduction event, a single successful one or multiple introductions of the same haplotype. Previous studies reported that H8 haplotype was typically found in the Mediterranean area included countries such as Cyprus, France, Greece, Italy, Spain, and Turkey (El- Mergawy *et al.*, 2011). As such, this means that *R. ferrugineus* found in Terengganu may be originated from Mediterranean area. A recent study showed that the *R. ferrugineus* at Fujian, China could have originated from Malaysia and Thailand due to the discovery of the same H8 haplotype (Wang *et al.*, 2015). Therefore, we can hypothesize that *R. ferrugineus* populations in Terengganu are originated from Mediterranean and may be closely related to populations from China.

**Conclusion and recommendations**

The mitochondrial cytochrome c subunit1 (CO1) gene was proven to be effective for to differentiate *R. ferrugineus* from similar *Rhynchophorus* species and for the identification of *R. ferrugineus*. The PCR amplification products of 600bp for *R. ferrugineus* were produced and revealed that different individuals of *R. ferrugineus* from districts of Terengganu did not shown any genetic variation, and can be concluded that they are similar in genetic content. As an invasive species, high similarity in genetic content of *R. ferrugineus* population is possible as the various populations may have originated from a single founder population. In addition, it can be concluded that *R. ferrugineus* populations in Terengganu originated from the Mediterranean as the single haplotype obtained in this study matches the H8 haplotype which is obtained from *R. ferrugineus* populations in the Mediterranean countries. Nevertheless, further studies must be proceeded in order to investigate other possible invasive pathways of *R. ferrugineus*. Additionally, more samples of *R. ferrugineus* should be obtained from other states in Malaysia to compare variation within and among the different states' *R. ferrugineus* populations. More studies are recommended to be conducted by using different molecular markers such as cytochrome b in order to gather more information on the genetic variations in *R. ferrugineus* populations.

**References**


Abraham, V.A., Al Shuaibi, M.A., Faleiro, J.R., Abozuhairah, R.A. & Vidyasagar, P.S.P.V. (1998). An integrated management approach for red palm weevil *Rhynchophorus ferrugineus* Oliv. a key pest of date palm in the middle east. *Journal of Agricultural Sciences* 3: 77-83.

Avise, J.C. (1994). *Molecular Markers, Natural History and Evolution*. New York: Chapman & Hall.

Black, W.C.I.V, Baer, C.F., Antolin, M.F. & DuTeau, N.M. (2001). Population genomics: genome-wide sampling of insect populations. *Annual Reviews Entomology* 46: 441-69.

Dlugosch, K.M. & Parker, I.M. (2008). Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Molecular Ecology* 17: 431-449. doi: 0.1111/j.1365-294X.2007.03538.x

El-Mergawy, R.A.A.M., Faure, N., Nasr, M.I., Avand-Faghih, A., Rochat, D. & Silvain, J. (2011). Mitochondrial genetic variation and invasion history of red palm weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae), in Middle-East and Mediterranean Basin. *International Journal of Agriculture Ture & Biology* 13: 631-637

EPPO (European and Mediterranean Plant Protection Organization). (2008). *Rhynchophorus ferrugineus*. *EPPO Bulletin* 38: 55-59. doi: 10.1111/j.1365-2338.2008.01195.x

Faleiro, J. (2006). A review of the issues and management of the red palm weevil *Rhynchophorus ferrugineus* (Coleoptera:Rhynchophoridae) in coconut and date palm during the last one hundred years. *International Journal of Tropical Insect Science* 26: 135–154.

Hussain, A., Rizwan-ul-Haq, M., Al-Jabr, A.M. & Al-Ayied, H.Y. (2013). Managing invasive populations of red palm weevil: A worldwide perspective. *Journal of Food, Agriculture & Environment* 11(2): 456-463.

Ju, R.T., Wang, F., Wan, F.H. & Li, B. (2011). Effect of host plants on development and reproduction of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae). *Journal of Pest Science* 84: 33-39.

Malumphy, C. & Moran, H. (2009). Red palm weevil *Rhynchophorus ferrugineus*. *The Food and Environment Research Agency (Fera)*: 1-4.

Mostoskey, U.V., Padgett, G.A., Stinson, A.W., Brewer, G.J. & Duffendack, J.C. (2000). Canine Molecular Genetic Disease, Michigan State University.

Murphy, S.T. & Briscoe, B.R. (1999). The red palm weevil as an alien invasive: biology and the prospects for biological control as a component of IPM. *Biocontrol News and Information* 20(1): 35-46.

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. ferrugineus* (Olivier 1790), and reveal the global extent of both. *Plos One* 8(10): e78379. doi:10.1371/journal.pone.0078379

Simon, C., Frait, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* 87(6): 651-701.

USDA (United States Department of Agriculture). (2006). What is a genetic marker? *Why We Care About Genetics* 5.

Vignal, A., Milan, D., Cristobal, M.S., Eggen, A. (2002). A review on SNP and other types of molecular markers and their use in animal genetics. *Genet. Sel. Evol. 34*: 275-305. doi: 10.1051/gse:2002009

Wahizatul, A.A., Zazali Chik, Abdul Rahman, A.R. & Nurul 'Izzah A.G. (2013). A new invasive coconut pest in Malaysia: the red palm weevil (Curculionidae: *Rhynchophorus ferrugineus*). *The Planter* 89(1043): 97-110.

Wang, G.H., Zhang, X., Hour, Y.M. & Tang, B.Z. (2015). Analaysis of the population genetic structure of *Rhynchophorus ferrugineus* in Fujian, China, revealed by microsatellite loci and mitochondrial COI sequences. *Entomologia Experimentalis et Applicata* 155: 28-38. doi: 10.1111/eea.12282

Table 1: Different haplotype of *R. ferrugineus* from different countries (El-Mergawy *et al.*, 2011).

| Haplotype | 3 | 15 | 43 | 54 | 57 | 78 | 81 | 88 | 94 | 102 | 114 | 174 | 216 | 233 | 300 | 303 | 318 | 354 | 363 | 372 | 381 | 405 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **H1** | A | T | G | G | A | A | T | T | A | G | A | G | G | G | A | A | A | C | G | G | T | C |
| **H2** | A | T | G | G | A | T | T | C | G | G | A | G | G | G | A | A | A | C | G | G | T | C |
| **H3** | A | T | A | G | A | A | T | T | A | G | A | G | G | G | A | A | A | C | G | G | T | C |
| **H4** | A | T | G | G | A | A | T | T | A | G | A | G | G | A | A | A | A | C | G | G | T | C |
| **H5** | A | T | G | G | A | A | T | T | A | G | A | A | G | G | A | A | A | C | G | G | T | C |
| **H6** | A | T | G | G | A | A | T | C | G | G | A | G | G | G | G | A | A | C | G | G | T | C |
| **H7** | G | C | G | A | G | G | T | T | A | A | G | G | G | G | A | A | A | T | A | G | C | C |
| **H8** | A | C | A | G | G | A | C | T | G | A | A | A | A | G | A | G | G | C | G | A | T | T |

Table 2: Similarity of haplotypes among all the 30 samples of *R. ferrugineus* with haplotypes 8 (H8).

| Haplotype | 57 | 78 | 81 | 88 | 94 | 102 | 114 | 174 | 216 | 233 | 300 | 303 | 318 | 354 | 363 | 372 | 381 | 405 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **H8** | G | A | C | T | G | A | A | A | A | G | A | G | G | C | G | A | T | T |
| **Kuala Terengganu** | G | A | C | T | G | A | A | A | A | G | A | G | G | C | G | A | T | T |
| **Kuala Nerus** | G | A | C | T | G | A | A | A | A | G | A | G | G | C | G | A | T | T |
| **Setiu** | G | A | C | T | G | A | A | A | A | G | A | G | G | C | G | A | T | T |
| **Besut** | G | A | C | T | G | A | A | A | A | G | A | G | G | C | G | A | T | T |
| **Marang** | G | A | C | T | G | A | A | A | A | G | A | G | G | C | G | A | T | T |
| **Dungun** | G | A | C | T | G | A | A | A | A | G | A | G | G | C | G | A | T | T |