

Haplotype Analysis and Phylogeny of *Oryzaephilus surinamensis* Populations from Four Regions in Peninsular Malaysia

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ABSTRACT

The sawtoothed grain beetle, *Oryzaephilus surinamensis*, is a secondary pest that damages rice products and other stored grains. Analysis based on the *cytochrome oxidase subunit I (COI)* sequences data, the number of haplotypes (Hap) (n), haplotype diversity (Hd), haplotype network, genetic distance, and phylogeny between *O. surinamensis* populations from four regions (small-scale), viz. the northern area (Seberang Perai), middle area (Klang), southern area (Pasir Gudang), and east coast (Kuantan) of Peninsular Malaysia, as model sampling locations, were obtained. A total of five haplotypes were detected in all the test populations, two shared (Haplotype 1 and Haplotype 3) and three unique haplotypes (Haplotype 2, Haplotype 4, and Haplotype 5) with haplotype diversity value, $Hd = 0.6789$ were recorded. Furthermore, the neighbour-joining (NJ), maximum parsimony (MP), and Bayesian inference (BI) trees showed a mixture of individuals from all regions in Peninsular Malaysia (Haplotype 1 to Haplotype 4), except Haplotype 5, which was

grouped with foreign populations that inherited similar haplotype with those of the European samples. This study assumed a mixture of populations presumably due to human activities and related explicitly to the exportation and importation of rice products across regions. This information is vital for strategising the control management of this pest species to reduce rice storage losses.

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INTRODUCTION

The sawtoothed grain beetle, *Oryzaephilus surinamensis*, is one of the main pests affecting stored grain worldwide (Hashem et al., 2012). This species also has been recorded as the most abundant pest species at a few Klang rice warehouses (Syarifah Zulaikha et al., 2018). In cereal grain commodities, *O. surinamensis* is a secondary pest that infests grains that are already infested by primary pests, such as *Sitophilus* spp. or *Rhyzopertha dominica* (Trematerra et al., 2012). The adults and larvae of *O. surinamensis* infest various food sources, such as rice, beans, cereals, legumes, flour, and dried fruits (Mowery et al., 2002). Globalisation and international trading activities of commodities have caused the widespread distribution of stored grain insect pests worldwide, especially in the hot climate regions, where the climate is conducive to the growth and development of the pests (Rees, 2004).

Many commercial and synthetic pesticides such as fumigants (Lee et al., 2003) and contact insecticides (Agrafioti & Athanassiou, 2018) have been applied to manage and control the infestation of the stored grain pests. However, long term usage of pesticides would be resulting in residues that will affect the grain quality, human health, and environment (Gahukar & Reddy, 2018). Furthermore, the continuous application of pesticides also induces resistance in this important storage pest, leading to difficulties in its control (Athie & Mills, 2005; Lorini et al., 2007). Thus, a better management process has to be utilised,

for example, by using physical (Vincent et al., 2009) and biological (Aman & Yaakop, 2013; Schöller, 2010) methods to manage the storage insect pests below the economic injury level. Some examples of the physical method are by manipulating the moisture content gradient and ventilation to affect the movement of *O. surinamensis*, where the ventilated wet grains would capture a greater proportion of *O. surinamensis* than dry, ventilated grains (Collins & Conyers, 2009). Meanwhile, *Cephalonomia tarsalis* (Hymenoptera: Bethyilidae) could suppress the *O. surinamensis* populations (Eliopoulos, 2019) and be an effective biological control method.

In planning an effective management program for this pest species, the population distribution must be understood for strategising protocol. Thus, the molecular approach using haplotype analysis is one of the methods to investigate the status of the pest species distribution (genetic distribution or gene flow) and the relationships among and between populations (Palraju et al., 2018). For example, one of the molecular studies reported on *O. surinamensis* showed no genetic differences in using 16S ribosomal DNA. However, the genetic separation was revealed by using AFLP and two mutations at the *COI* (Sharaf et al., 2013). On the other hand, *Oryzaephilus surinamensis* showed no genetic differences in using 16S ribosomal DNA. Nevertheless, the genetic separation did occur, as revealed by using AFLP and two mutations at the *COI*. Other than that, Govindaraj et al. (2014) showed genetic variations between two populations

of *O. surinamensis* by using RAPD markers due to host characteristics. Based on the positive results of AFLP, RAPD, and COI markers, haplotype analysis using *COI* was performed in this study to investigate the distribution of *O. surinamensis* and relationships among populations sampled from different locations throughout the country.

With the current rising demand for effective pest control of storage food products to ensure food security and safety, it is better to understanding the insects' relationships among populations across regions. Thus, it will contribute significantly to improving the current management strategy for controlling major insect pests. Hence, this study aims to measure the

haplotype analysis, illustrate the haplotype network, and construct a phylogeny of *O. surinamensis* populations from Peninsular Malaysia.

METHODS

Sampling Sites

Fresh samples of *Oryzaephilus surinamensis* were collected from four different localities or regions, i.e. Klang (middle area), Kuantan (east coast), Seberang Prai (northern area), and Pasir Gudang (southern area) in Peninsular Malaysia, from June 2016 until July 2017 (Figure 1). This sampling method is considered model sampling for small-scale regions because the distance between sampling sites is less than 1,000 km apart.



Figure 1. Localities of *Oryzaephilus surinamensis* collected from four different regions in Peninsular Malaysia

Insect Sampling

A total of 20 individuals of *O. surinamensis* were successfully collected from four regions, comprising five individuals from each sampling site. According to Goodall-Copestake et al. (2012), a total of five individuals from each population are sufficient for the first assessment of genetic diversity. Active sampling was applied throughout the sampling process. Fresh samples were kept in 100% alcohol to prevent DNA degradation and avoid contamination. The samples were identified until species level using a stereomicroscope (Zeiss Stemi DV4, Germany) and by referring to the key to species by Rees (2004). Further action was taken by keeping the samples in the freezer at -20°C for molecular work. The data on temperature and humidity have been standardised in all the warehouses.

DNA Extraction and PCR Amplification

DNA was extracted from each sample using the NucleoSpin® DNA Insect Kit (MACHEREY-NAGEL, Germany) according to the manufacturer's instruction. The whole body of *O. surinamensis* was used to maximise the DNA volume. Forward and reverse primers chosen were LCO14905'-GGTCAACAAATCATAAAGATATTGG-3' and HC02198 5' - TAAACTTCAGGGTGACCAAAAATCA-3' (Folmer et al., 1994) to amplify the *cytochrome oxidase subunit I (COI)* using a Mastercycler Nexus (Eppendorf, United States of America). The PCR was performed by using a mixture of 12.5 µl of Green Mastermix

(Promega, United States of America), 7.5 µl of ddH₂O, 3 µl of template DNA, and 1.5 µl of primer region each from forward and reverse site with the total volume was 25 µl. The PCR process completed each cycle by undergoing a few steps included 3 min of 95°C pre-denaturation, 30 cycles of denaturation for 30 sec at 95°C, 1 min of annealing at 47°C, 30 sec of elongation at 72°C and 10 min of final elongation at 72°C (Halim et al., 2017, 2018). All the PCR products of *O. surinamensis* were sent to Apical Scientific (Malaysia) for sequencing.

Sequences Editing, BLAST, BOLD Analyses, and Sequences Alignment

COI sequences were edited through Sequencher v4.1 software by combining forward and reversed primer and forming a complete sequence. The Barcode of Life Data System (BOLD) (Ratnasingham & Hebert, 2007) and Basic Local Alignment Search Tool (BLAST) (Benson et al., 2013) were used for a comparison between both sample and database sequences based on its evenness percentage and small probability value E (Krauthammer et al., 2000). ClustalW and MEGA7 were applied to align the sequences with the outgroup *Oryzaephilus mercator* (MG458965.1).

Haplotype Analysis and Haplotype Network

Haplotype analysis was performed for the *COI* sequences using DNA Sequence Polymorphism (DnaSP) software, v6 (Rozas et al., 2017). Haplotype diversity (*H_d*) and the number of haplotypes (*n*)

were two parameters analysed for the haplotype analysis. For the minimum-spanning network (MSN), Network 4.6.1.2 was used (Bandelt et al., 1999).

Phylogenetic and Genetic Distance Analyses

For phylogenetic analysis, three sequences from the Genbank database were selected, which were KM446924.1 (Germany), KU4942022.2 (France), and KC129180.1 (Finland) to aid the analysis. The neighbour-joining tree was constructed using PAUP* v4.0b10 for 1,000 replications using the Kimura two-parameter (K2P) model. Maximum parsimony (MP) tree was performed using PAUP* v4.0b10 with 100 stepwise additions. Bayesian inference was constructed using MrBayes 3.2 (Ronquist et al., 2012). jmodelTest 3.7 (Darriba et al., 2012) was run to get the Akaike's Information Criterion (AIC) calculation

to select the best model to construct the Bayesian tree. The analysis was run using 1 million generations, and Bayesian tree topology is considered entirely constructed when the posterior probability was <0.01. The phylogenetic trees were visualised in Tree view 3.0, with *Oryzaepphilus mercator* (MG458965.1) as an outgroup. Genetic distances within and between populations were performed using Kimura 2-Parameter (K2P) in MEGA7 software.

RESULTS

Sequence Variation

A total of 20 samples of *O. surinamensis* were successfully extracted in this study, and 620 bp of the *COI* was successfully amplified from each individual. Each sample was recorded in the Genbank, and their accession numbers were listed in Table 1 according to their locations.

Table 1

Information on accession number of *Oryzaepphilus surinamensis* from the Genbank

Species	Code	Accession number	Sampling site
<i>Oryzaepphilus surinamensis</i>	JASAKlang1	MH587653	Klang, Selangor
<i>Oryzaepphilus surinamensis</i>	JASAKlang2	MH587654	Klang, Selangor
<i>Oryzaepphilus surinamensis</i>	JASAKlang3	MH587655	Klang, Selangor
<i>Oryzaepphilus surinamensis</i>	JASAKlang4	MH587656	Klang, Selangor
<i>Oryzaepphilus surinamensis</i>	JASAKlang5	MH587657	Klang, Selangor
<i>Oryzaepphilus surinamensis</i>	JYAJohor1	MH587658	Pasir Gudang, Johor Bahru

Table 1 (Continued)

Species	Code	Accession number	Sampling site
<i>Oryzaephilus surinamensis</i>	JYAJohor2	MH587659	Pasir Gudang, Johor Bahru
<i>Oryzaephilus surinamensis</i>	JYAJohor3	MH587660	Pasir Gudang, Johor Bahru
<i>Oryzaephilus surinamensis</i>	JYAJohor4	MH587661	Pasir Gudang, Johor Bahru
<i>Oryzaephilus surinamensis</i>	JYAJohor5	MH587662	Pasir Gudang, Johor Bahru
<i>Oryzaephilus surinamensis</i>	MISCPenang1	MH587663	Seberang Prai, Pulau Pinang
<i>Oryzaephilus surinamensis</i>	MISCPenang2	MH587664	Seberang Prai, Pulau Pinang
<i>Oryzaephilus surinamensis</i>	MISCPenang3	MH587665	Seberang Prai, Pulau Pinang
<i>Oryzaephilus surinamensis</i>	MISCPenang4	MH587666	Seberang Prai, Pulau Pinang
<i>Oryzaephilus surinamensis</i>	MISCPenang5	MH587667	Seberang Prai, Pulau Pinang
<i>Oryzaephilus surinamensis</i>	EMAKuantan1	MH587668	Kuantan, Pahang
<i>Oryzaephilus surinamensis</i>	EMAKuantan2	MH587669	Kuantan, Pahang
<i>Oryzaephilus surinamensis</i>	EMAKuantan3	MH587670	Kuantan, Pahang
<i>Oryzaephilus surinamensis</i>	EMAKuantan4	MH587671	Kuantan, Pahang
<i>Oryzaephilus surinamensis</i>	EMAKuantan5	MH587672	Kuantan, Pahang

Five haplotypes with 11 bp were defined from four populations of 20 individuals of *O. surinamensis* (excluding outgroup). Only the Kuantan population had three haplotypes (Hap 1, Hap 3, and Hap 5) in which Hap 5 was the unique haplotype. At the same time, Hap 1 and Hap 3 were shared between the Klang and Penang populations. In addition, both Klang and Johor samples have unique haplotypes (Hap 2 and Hap 4).

The haplotype diversity (Hd) was 0.6789 (Table 2).

In Figure 2, Hap 5, represented by two individuals from Kuantan, showed the most extended mutational steps compared to the other haplotypes. Meanwhile, Hap 1 showed the largest circle, a feature shared by three populations, i.e. Klang, Seberang Prai, and Kuantan, continued by Hap 3 as recorded from the sample populations collected from

Table 2

Segregating sites (11bp) in 620 bp of COI marker defining five haplotypes of Oryzaephilus surinamensis populations

Haplotypes	Nucleotides positions				Locality			
					1 Klang	2 Pasir Gudang	3 Seberang Prai	4 Kuantan
	1 2	2 3	5 5					
	2	4						
	6 0	5 0	1 4					
	0	4						
	6 8	8 1	8 4	7 0				
	9	4	1					
Hap_1	T G	A T	C A	A A	4		4	
	A	G	A				2	
Hap_2	G A	1			
	G		. . .					
Hap_3	. . .	G		4	1	
				1	
Hap_4	. . .	G		1		
	G . .					
Hap_5 C	T G	G G			2	
	. . .	A	T					

Note. A = Adenine; C = Cytosine; G = Guanine; T = Thymine

Pasir Gudang, Seberang Prai, and Kuantan. On the other hand, both Hap 2 and Hap 4 have the smallest circle because each was represented by one individual only.

Genetic Distance and Phylogenetic Trees

The highest genetic distance was showed between Hap 2 - Hap 5 with 91%. Meanwhile, the lowest genetic distance was between Hap 1 - Hap 3 and Hap 3 - Hap 4 with 9%. Among all the haplotypes, Hap 5 showed a higher genetic distance with all the

other haplotypes, 64%, 91%, 73%, and 64%. In comparison, the combination of other haplotypes ranged between 9% - 45% (Table 3). From Figure 3, Pasir Gudang–Seberang Prai showed the nearest clade with the lowest genetic distance is 0.001. Meanwhile, Pasir Gudang–Kuantan and Klang–Kuantan showed that the farthest clade with genetic distance is 0.006 (Table 4).

Neighbour-joining (NJ) tree constructed showed a bootstrap value ranging between 50% - 100%. However, all individuals from each population were not successfully

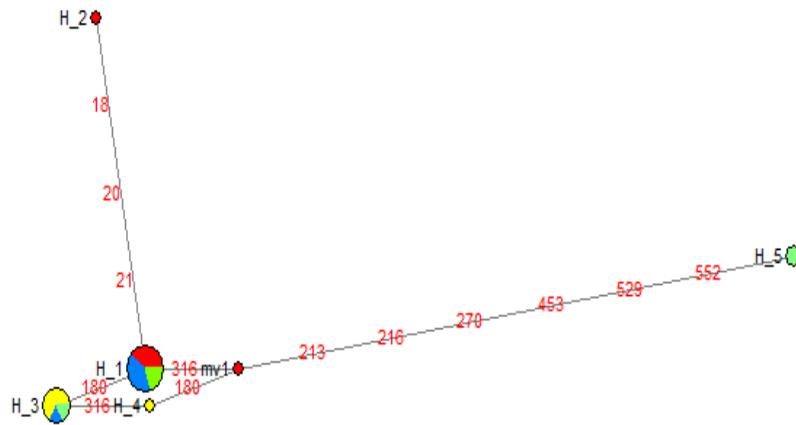


Figure 2. Haplotype network based on COI dataset

Table 3

Percentage of sequence differences between pairs of haplotypes

Haplotype	Distance (%)
Hap 1 - Hap 2	27
Hap 1 - Hap 3	9
Hap 1 - Hap 4	18
Hap 1 - Hap 5	64
Hap 2 - Hap 3	36
Hap 2 - Hap 4	45
Hap 2 - Hap 5	91
Hap 3 - Hap 4	9
Hap 3 - Hap 5	73
Hap 4 - Hap 5	64

grouped into a similar group or population. While two individuals, EMA 1 and EMA 5 from Kuantan, form a paraphyletic clade with other EMA individuals by 100% bootstrap value (Figure 3). Both EMA 1 and EMA 5 had seven similar nucleotides, which

caused them to be grouped and separated from others. The MP supported the defined separation of the Kuantan populations with 98% bootstrap value. Bayesian inference tree was constructed with a model of GTR+G through jmodelTest 3.7. The

topology showed EMA 1 and EMA 5 grouped with Germany, France, and Finland samples, which they located at the basal of the tree with posterior probability (pp) 0.98. These two individuals had separated with a new clade with pp = 0.89 and formed a subclade with pp = 0.80 (Figure 4).

Table 4
Genetic distance of *Oryzaephilus surinamensis* between populations

Location	Distance
Klang - Seberang Prai	0.002
Klang - Kuantan	0.006
Klang - Pasir Gudang	0.003
Pasir Gudang – Seberang Prai	0.001
Pasir Gudang - Kuantan	0.006
Seberang Prai - Kuantan	0.005

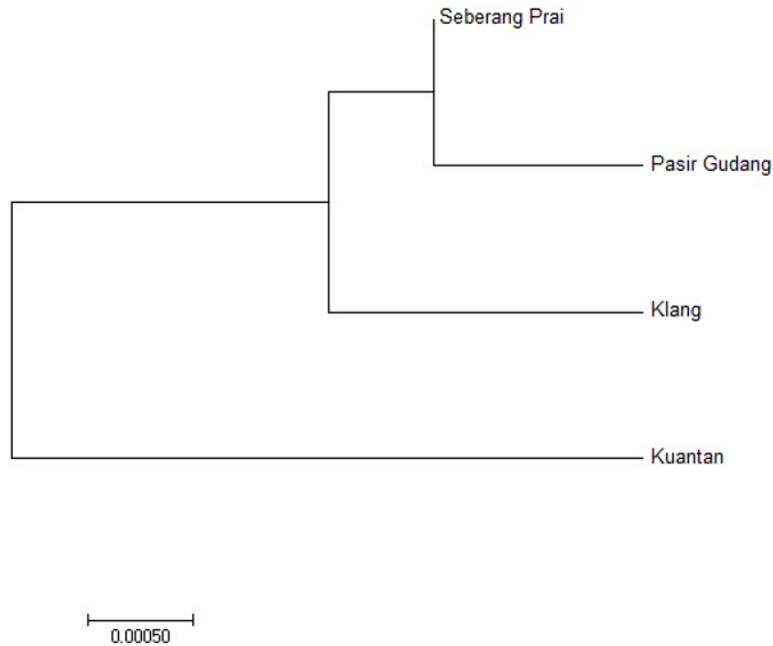
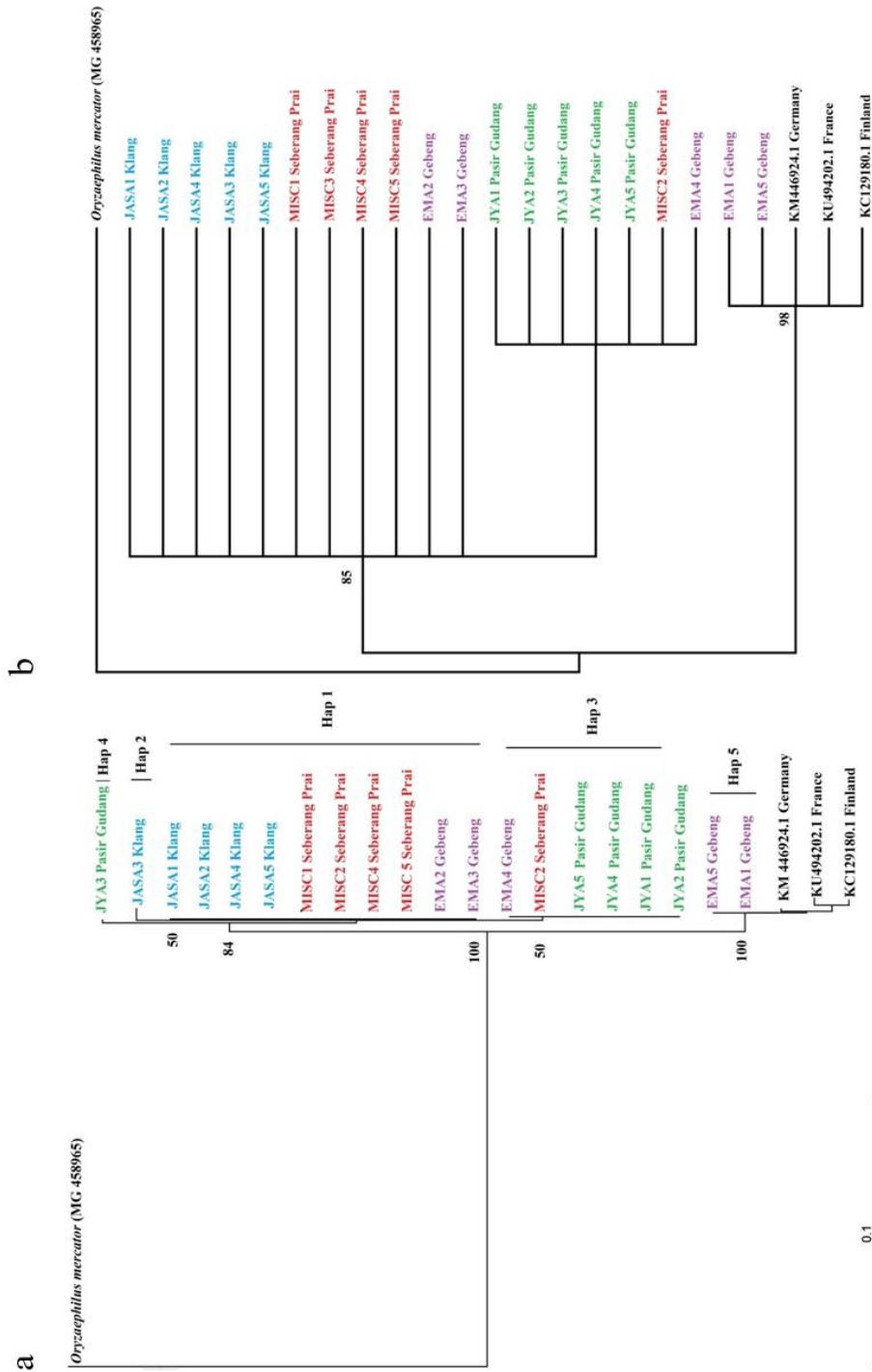


Figure 3. Population tree by haplotype sequence among four populations



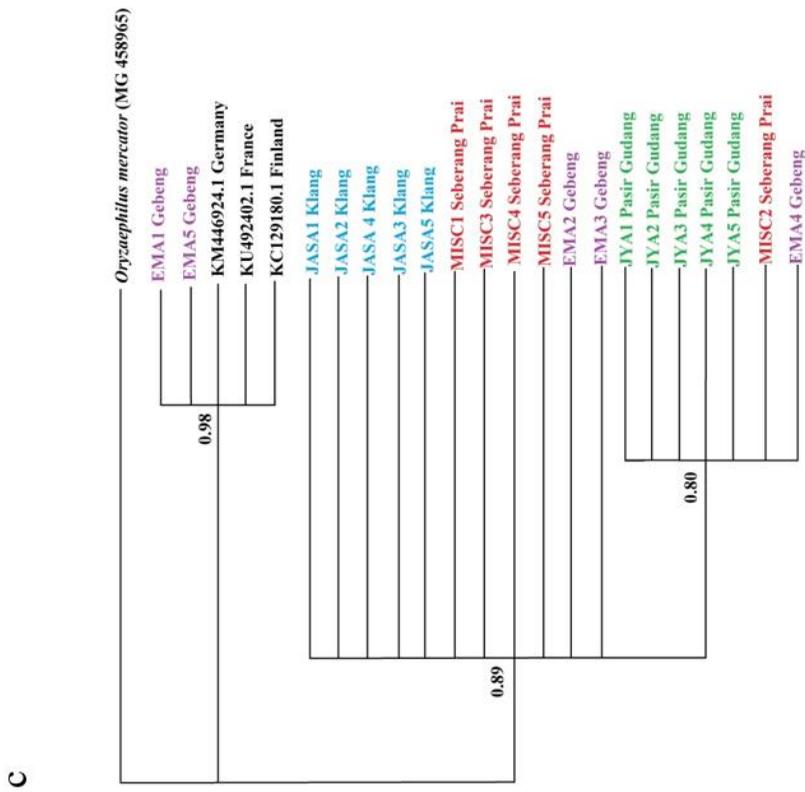


Figure 4. (a) The bootstrap tree resulted from neighbour-joining (NJ) analysis of *Oryzaephilus surinamensis* COI sequences data based on 1,000 replications; (b) The bootstrap tree resulted from maximum parsimony (MP) analysis of *O. surinamensis* COI sequences data based on 1,000 replications. Values above the lineages are the bootstrap values; (c) The Bayesian tree resulted from Bayesian inference (BI) analysis of *O. surinamensis* COI sequences data. Values at the internal nodes are the posterior probabilities (pp)

DISCUSSION

The mtDNA has been widely used to detect the genetic diversity, genetic differentiation, and population structure of organisms, e.g. study by Ambrose et al. (2012), Annan et al. (2007), Dyer et al. (2008), and Wellenreuther et al. (2011). The mtDNA *COI* marker efficiently measures the genetic distance of test populations either within or between the populations. Notably, several studies have suggested that *COI* is an ideal marker for examining interspecific or intraspecific phylogenetic relationships among insects (Ahmed et al., 2009; Sole et al., 2008). Furthermore, this marker has also been used widely as the barcoding marker in identifying and discriminating storage pest species for Integrated Pest Management (IPM) purposes (Ghazali et al., 2014; Halim et al., 2017, 2018). Therefore, despite the small number of samples implemented in the genetic diversity analyses, the data were sufficient for genetic diversity analysis. Furthermore, a study by Amzati et al. (2018) and Gollner et al. (2016) also showed that several samples implemented for the analysis did not significantly affect the study results.

The geographical factors, such as landscape, latitude, longitude, and environmental factors (temperature and precipitation), can affect a species' genetic and population structures (Pauls et al., 2013; Wellenreuther et al., 2011). The populations located at the western coast of Peninsular Malaysia were hypothesised to differ distinctly from those of the eastern coast because of the physical barrier created by

the Main Range (Banjaran Titiwangsa) as in many animal species. So, the exact condition will happen for the *O. surinamensis* from four geographical ranges at the west and east-coast of Peninsular Malaysia. Several studies on weak-flying insects support this information. For example, Cheng et al. (2014) reported on various termite species populations, *Coptotermes gestroi*, that exhibited haplotypes differences between the west and east coasts of Peninsular Malaysia (area sites) using *COI* and 16S sequences.

A study by Sum et al. (2014) also recorded the distinct haplotype separation between the northern and central populations of *Anopheles sinensis* that were geographically isolated by the mountain ranges of Gunung Tahan and Banjaran Titiwangsa (Sankalakhiri Range). Notably, most beetle species are generally weak flyers (Tuda et al., 2014). The adults of *O. surinamensis* could hardly fly anywhere far (Heaps, 2006). Thus, this study predicts that all individuals from the same population are grouped and have similar haplotype number. However, our finding contradicted the hypothesis, in which all individuals were found to be pretty well mixed up between populations. It is strongly indicated that some other factors besides the physical barrier had likely contributed to this situation. Based on this finding, that situation is not related to insect storage pests. In contrast, the distribution of the species is probably affected by the human or commodity movement (S. Yaakop, personal communication, July 10, 2017).

The results showed that the mixture of populations had likely occurred between the four locations for most of the study samples (Figure 4). The results have been supported by NJ, MP, and BI trees, which showed that all populations from west-central, southern, northern, and eastern areas were mixed. This observation was also supported by the high to low bootstrap values and posterior probabilities. The maximum distance between two locations is about 630 km, for example, on Seberang Prai-Pasir Gudang. This result was supported by Mazzi and Dorn (2012) in their study of the herbivorous insect pests for contemporary agro ecosystems and by Wei et al. (2015) for the oriental fruit moth, *Grapholita molesta* infesting on stone and pome fruits, with observations that genetic exchange may happen due to human-mediated dispersals and transport by the host plants over a huge area of geographic distance.

At all the warehouses in this study, there were active rice exportation and importation activities from one warehouse to another within the region. Likewise, human activities, migration, and gene flow were the main factors affecting the genetic study and population structure besides climate change and other environmental and ecological factors. The *O. surinamensis* also a widespread pest due to the infestation that involves manufacturing, storage, and retail phases (Nurul-Huda & Noor-Amni, 2020). Here, human activities were concluded as one of the main reasons for the population mixing of the pest species. The haplotype analysis and haplotype network (Table 2; Figure 2) showed that the Klang, Seberang

Prai, and Kuantan populations shared similar haplotypes for specific individuals.

In our study, this haplotype appeared in four individuals from Klang, four from Seberang Prai, and two from Kuantan. Furthermore, Hap 3 was also shared between three locations, i.e. Pasir Gudang, Seberang Prai, and Kuantan. Thus, the genetic exchange had likely occurred between the populations of the eastern (Kuantan) and western parts of Peninsular Malaysia (Seberang Prai, Klang, and Pasir Gudang) (Table 3). Hap 5 indicated that it was split from the old population with one ancestral haplotype and carried the alleles that were inherited together with the foreign samples (European populations) caused by mutations (Doorenweerd et al., 2020).

In NJ, MP, and BI topology, these groups formed a polytomy showing unresolved relationships between them (Figure 4). Interestingly, two samples from Kuantan presented one unique haplotype, which was formed and separated from the main clade of all the samples from all populations. It is suggested that the information of two individuals from Kuantan was unique and distinct from other samples or haplotypes (Table 2). Clustering of two individuals EMA1 and EMA5, with the samples from GenBank could indicate that these alleles were inherited together with the samples from Finland, Germany, and France (European). It showed that *O. surinamensis* was not native from Malaysia and was described by Linnaeus in 1786 in Suriname, in the northern part of South America (Champ & Dyte, 1976). However, it might have originated from ancient Greece based on the paleontological record in 7000 BC

due to the presence of stored product stock such as wheat, etc. (Llobera, 2002).

Generally, different varieties of rice stocks from several countries worldwide were imported and delivered to the Malaysian retailers through several harbours in Peninsular Malaysia. From there, the stock would be sorted out and divided into several warehouses in the country. In this regard, it is very imperative to obtain accurate information on to the origin of the insect populations in international trade to effectively manage and control the pest infestations related to our food security and safety. Therefore, it is also supported by Fleurat-Lessard and Pronier (2006) to apply genetic data through random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique to understand the population level of pest species towards international trade.

CONCLUSION

This study has successfully presented the phylogeny, haplotype data, and haplotype network of *Oryzaephilus surinamensis* populations based on *COI* sequences from four localities or regions in Peninsular Malaysia. Five haplotypes were obtained, of which Hap 5 showing uniqueness, inherited together or shared with the foreign samples (European populations). The mixture of the populations revealed a close relationship of this species across the east and west coast populations. It was most probably due to human activities by rice exportation and importation from one warehouse to another within the regions. This study

could provide the initial and fundamental data with the potential to be used in IPM to strategise the control management of important pest species. Additional molecular markers and more individual samples per locality are suggested to be added for more comprehensive data. Implementing the population genetic study is also highly necessary to draw a solid conclusion soon.

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