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Full Length Research Paper

Genetic variation studies in *Oryctes rhinoceros* (L.) (Coleoptera: Scarabaeidae) from oil palm plantations using random amplified microsatellite (RAMs) markers

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Randomly amplified microsatellite markers were used to study the genetic variation among six populations of *Oryctes rhinoceros* L. which were collected from oil palm plantations in Selangor, Perak, Pahang and Medan. Samples were collected using light and pheromone trapping for the purpose of obtaining two populations per site study. Thirty individual beetles per population were screened using seven randomly amplified microsatellite primers. Beetles were not attracted to light traps at Pahang and Medan. This resulted in only pheromone populations being caught there. Distances calculated based on the similarity coefficient of Nei and Li (1979) ranged between 0.422 and 0.736. Seventy eight reproducible loci were generated using the seven primers and all the loci were polymorphic. The dendrogram constructed produced two major clusters. Based on the dendrogram, the clusterings were observed to be influenced by preference to trapping system as well as geographical distance. The separation of clustering between Perak Pheromone (PP) and Perak Light (PL) is important as it gives rise to the possibility for the presence of two groups of *O. rhinoceros* based on their preference toward light and pheromone trap. However, further studies using codominant markers especially single locus DNA microsatellite markers are required to understand the population genetic structure and to further validate the presence of a cryptic species complex.

Key words: *Oryctes rhinoceros*, RAMs, genetic variation.

INTRODUCTION

The Malaysian oil palm industry has observed rapid development ever since its establishment since 1911. This golden crop of Malaysia has contributed a total income of RM49.6 billion in the year 2009. Humid tropical condition and various environmental factors have favored the infestation of various pests in oil palm plantations in Malaysia. *Oryctes rhinoceros* or commonly known as rhinoceros beetle has caused major problems to plantations in both the east and the west coast of Peninsular Malaysia. A national survey on *O. rhinoceros* by Norman and Basri (1997) revealed the occurrence of this pest within one to six months after replanting in most estates. The enforcement of the zero burning concept further aggravated the situation (Ariffin and Basri, 2000). *O. rhinoceros* has been observed to inflict serious damage on young palms in replanting sites which further led to crop loss.

Past research has produced supporting statistics that indicate the rates of damage by *O. rhinoceros*. In

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Abbreviations: RAMs, Randomly amplified microsatellite; **MP,** Medan Pheromone ; **PL,** Perak Light; **SL,** Selangor Light; **PP,** Perak Pheromone; **PaP,** Pahang Pheromone; **PCR,** polymerase chain reaction; **ISSR,** intersimple sequence repeat.

Malaysia, in 1995, a survey showed that 25% of 180 068 ha, of young palms were attacked (Liau and Ahmad, 1991). More than 15% reduction in canopy size was observed by Samsuddin et al. (1993). The loss of leaves lead to reduced photosynthetic activity, delayed plant maturity, reduced fruit bunch size and finally, crop loss. Chung et al. (1999) have also reported that, damage by *O. rhinoceros* could cause an average of 40% crop loss in the first year of harvesting.

To date, pheromone trapping has gained popularity among plantation managements due to its efficiency and economical value (Norman and Basri, 1995). A species specific semiochemical called aggregation pheromone containing ethyl-4-methyloctanoate is used in trapping (Hallet et al., 1995). However, not all the populations of *O. rhinoceros* in the field are attracted to the pheromone. This led to a question on the possible presence of a cryptic species complex in rhinoceros beetles. A cryptic species complex is a group of species that are reproductively isolated from one another but are not morphologically distinguishable (Bickford et al., 2007). The individual species within the complex can only be separated using non-morphological data, such as from molecular marker analysis.

Random amplified microsatellites are a popular and promising genetic marker system that has produced many beneficial findings in the field of molecular genetics. This marker is also known as the intersimple sequence repeat (ISSR) marker. The randomly amplified microsatellite is generated from a single-primer polymerase chain reaction amplification (PCR) reaction (Zietkiewicz et al., 1994). The primer is designed from dinucleotide, trinucleotide, tetranucleotide or pentanucleotide repeat motifs. One to three nucleotide anchors are used at the 5' end to prevent strand slippage during PCR amplification (Crawford et al., 2001). During PCR, genomic segments flanked by the inversely oriented and closely spaced repeats will be amplified and multilocus profiles with abundant polymorphism will be generated (Reddy et al., 1999). The objective of this study was to use randomly amplified microsatellite (RAMs) primers to amplify bands that could be used to study the genetic variation in *O. rhinoceros* populations from oil palm plantations.

MATERIALS AND METHODS

Materials

Life adult *O. rhinoceros* beetles were collected from oil palm replanting sites in four different locations which are Tennamaram Estate in Selangor, Felcra Berhad in Perak, Kuantan Trading Plantations in Pahang and Paya Pinang Plantations in Medan, Indonesia. At each site, two populations of beetles were trapped; one using pheromone traps produced by Sime RB® pheromone and the other using light traps. Both traps were placed along the fringes of replanting sites and the distance between both traps was generally less then 15 m at all study sites. All the samples were stored in a -80°C freezer. Thirty individuals were analyzed from each respective trap.

DNA extraction

Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's protocol for animal tissue genomic DNA isolation with minor modifications. DNA was extracted from the thorax and head tissues to avoid contamination. The extracted DNA was quantified by visualization on 1% agarose gel (w/v) horizontal at 70 V for 45 min using 1X TBE buffer (0.045 M Tris-borate and 1 mM EDTA, pH 8). The electrophoresis product was stained in 0.1 µg/µl of ethidium bromide for 20 min. The DNA yield was estimated by using Hyper Ladder 1 (Bioline) as a reference. The DNA concentration and 260:280 nm absorbance ratios were also determined using a spectrophometer (Ultrospec III, Pharmacia). All extracted DNA were stored in a -20°C freezer.

Primer selection

A total of 15 RAMs primers designed by Kumar (2003) and Hoh (2006) were screened. Primers that amplified and produced informative banding patterns which are useful for identification of genetic variation within and among populations were chosen. Meanwhile, primers that do not produce distinct bandings were disregarded. The beetle populations were then screened using primers that were able to amplify genomic DNA during PCR and the observed banding patterns were analyzed.

DNA amplification

DNA amplification was done in a total volume of 10 µl consisting of 25 ng of genomic DNA, 2.5 mM $MgCl_2$, 1X PCR Buffer, 0.4 mM of each dNTPs, 0.3 - 0.5 µM of specifically optimized primer, 3U of *Taq* DNA polymerase (Promega, USA) and topped with deionized distilled water up to 10 µl. The mixture was overlaid with 3.5 µl of mineral oil. Amplifications were performed in a Techne TC-412 thermal cycler with an initial 3 min of predenaturation at 96°C, followed by 40 cycles of 20 s denaturation at 95°C, 20 s annealing at a specifically optimized temperature for each primer and 35 s extension at 68°C. A final extension step of 68°C for 5 min was included. 5 µl of the PCR product was electrophoresed on 2% horizontal agarose gel (w/v) at 70 V for 90 min depending on the size of the amplified fragments. A 100 base pair extended ladder (Fermentas) was used as a molecular weight standard. The products were further stained in 0.1 µg/µl ethidium bromide and subsequently visualized over UV light and captured using an Alpha®Imager 2200 (Alpha Innotech, USA) system.

Data analysis

Data scoring was done according to the presence/ absence criteria (1= presence of band; 0 = absence of band). Clear and polymorphic banding patterns were visually scored and faint bands were not included during scoring. Shannon's Information index (I) was calculated as I = Σpilog2pi, where pi is the frequency of a given band for each population (Lewontin, 1972). Based on the data, similarity coefficients were calculated across all possible pairwise comparisons of individuals both within and among populations. From that data, a pairwise distance matrix was compiled based on the similarity coefficient of Nei and Li (1979) using the RAPDistance version 1.04 software (Armstrong et al., 1995). The genetic

Table 1. Sequences of RAMs primers which successfully amplified *O. rhinoceros* DNA and their optimized conditions.

Primer	Primer sequence 5'-3'	Annealing temperature (°C)	Primer concentration (µm)
LR4	GCA CAT GCA R$(TG)_7$	55.0	0.3
LR5	GAT GCG ATR$(CA)_7$	55.0	0.5
LR7	KKV RVR V$(GA)_{10}$	53.0	0.4
BP10	KKD RDR D$(TC)_{10}$	43.6	0.4
BP11	KKY HYH Y$(CAG)_5$	50.0	0.4
SC3	NNN NNN NNN MMH RVH RV$(GTC)_4$	39.0	0.4
VJ1	NNN NNN NKK VRV RV$(CT)_{10}$	42.5	0.4

IUB code: K, G/T; V, A/C/G; R, A/G; M, A/C; H, A/C/T; Y, T/C; W, A/T; B, C/G/T; D, A/G/T; S,C/G; N,A/C/G/T.

Table 2. Number of loci generated by seven RAMs primers in 6 populations of *O. rhinoceros*.

Primer	Total reproducible bands	Total polymorphic band	Polymorphic loci (%)	Number of polymorphic loci according to *O. rhinoceros* population					
				SP	SL	PP	PL	PaP	MP
VJ1	7	7	100	7	6	7	6	7	0
SC3	9	9	100	7	7	7	9	5	9
LR4	15	15	100	8	8	9	9	8	8
LR5	13	13	100	11	9	11	9	10	0
LR7	9	9	100	3	6	6	8	5	4
BP10	14	14	100	11	11	7	9	7	10
BP11	11	11	100	7	8	6	8	7	7
Total	78	78		54	55	53	58	49	38
%Polymorphism				69.23	70.52	67.95	74.36	62.82	48.72

relationships were visualized by a dendrogram based on the distance values derived from similarity matrices (distance = 1 – similarity). The dendrogram was constructed using the unweighted pair group method with arithmetic averaging (UPGMA), employing the SAHN (Sequential, agglomerative, hierarchical and nested clustering) program from NTSYS-pc version 1.6 (Numerical Taxonomy and Multivariate Analysis System) (Rohlf, 1993).

RESULTS

Seven RAMs primers which gave clear and distinct banding patterns were used to investigate the genetic relationships and resolve the differences among six *O. rhinoceros* populations. Screening of the populations was repeated thrice and it gave highly reproducible results. Table 1 shows the RAMs primer sequences and their optimized PCR conditions which successfully amplified the genomic DNA and were reproducible. At each study site, sample collection was carried out simultaneously using both pheromone and light traps for duration of one to two months. Beetle samples were successfully collected in Selangor and Perak in both pheromone and light traps. However, in Pahang, Medan and Indonesia, beetles were only attracted to the pheromone trap. Therefore, only pheromone attracted samples were trapped at these sites and light attracted samples was not obtained. This resulted in a total of six populations, each containing 30 individuals being collected from the four study locations.

The number of loci generated by the seven RAMs primers in six populations of *O. rhinoceros* is shown in Table 2. A total of 78 reproducible loci were generated using the seven primers and all the loci were polymorphic. The number of polymorphic loci per primer varied between 7 for VJ1 and 15 for LR4. The overall banding profile showed that, the largest fragment was 2800 bp while the smallest fragment was 340 bp in size. The highest percentage of polymorphic loci was in the Perak Light (PL) population at 74.36%, while the Medan pheromone (MP) population had the lowest percentage of polymorphic loci at 48.72%.

An example of variation in banding profile that was observed using primer LR4 in beetles trapped in Perak using pheromone and light traps is illustrated in Figures 1 and 2. Polymorphism in the loci banding patterns is clearly visible at that location. The pairwise distance matrix compiled based on the similarity coefficient of Nei and Li (1979) for 6 populations of *O. rhinoceros* ranged between 0.422 and 0.736. This is illustrated in Table 3. The highest distance value (0.736) occurred between the

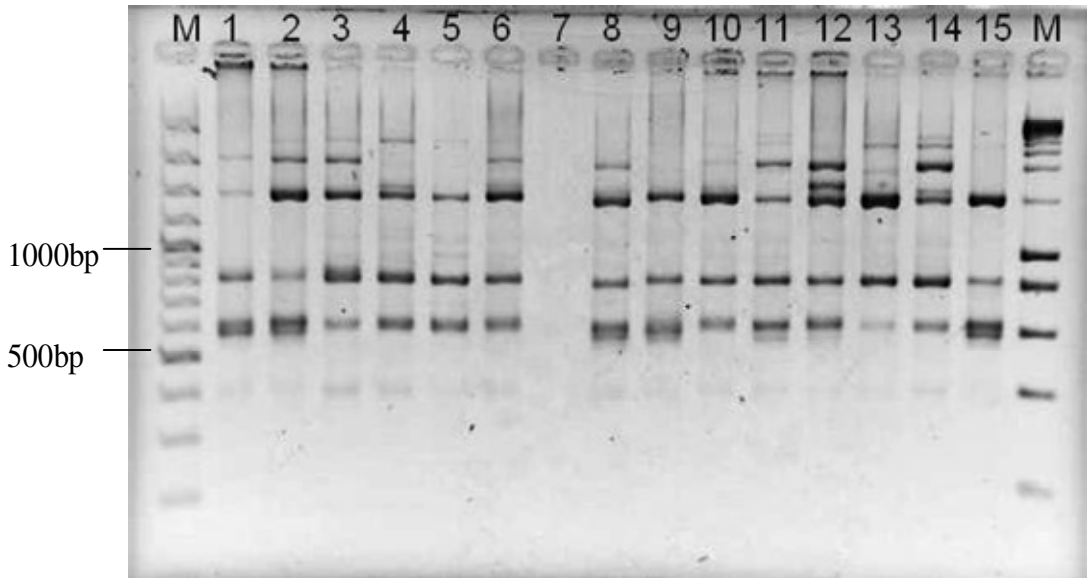

Figure 1. RAMs profile generated by primer LR4 in Perak pheromone individuals on 2% agarose gel using 1XTBE buffer. Lane M, 100bp extended (Fermentas) molecular weight marker; Lane 1 to 15: Individual beetles from Perak pheromone trap.

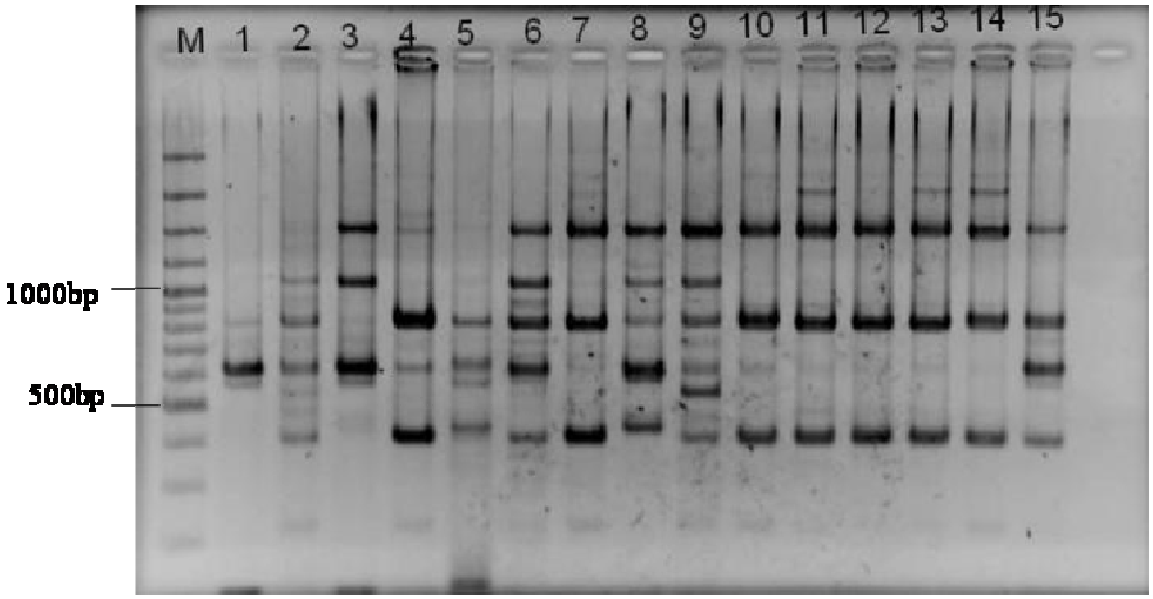

Figure 2. RAMs profile generated by primer LR4 in Perak light individuals on 2% agarose gel using 1X TBE buffer. Lane M: 100bp extended (Fermentas) molecular weight marker; Lane 1-15: Individual beetles from Perak light trap.

Medan Pheromone (MP) and the Selangor Light (SL) populations and the lowest distance value (0.422) was between the Selangor Light (SL) and the Selangor Pheromone (SP) populations. Table 4 shows the Shannon Information index value for the six populations of *O. rhinoceros*. The value of Shannon Information index ranged from 0.2476 (Medan Pheromone) to 0.3922 (Perak Light), with a general mean of 0.3265.

Looking at the dendrogram in Figure 3, two major clusters were observed. In the first major cluster, the Selangor Pheromone (SP) population and the Selangor Light (SL) population was grouped as a subcluster and the Perak Pheromone (PP) population produced an individual group. The second major cluster included the Perak Light (PL), Pahang Pheromone (PaP) and Medan Pheromone (MP) populations. The Perak Light (PL) and

Table 3. A pair wise distance matrix compiled based on the similarity coefficient of Nei and Li (1979) for 6 populations of *O. rhinoceros*.

	SP	SL	PP	PL	PaP	MP
SP	0.000					
SL	0.422	0.000				
PP	0.558	0.516	0.000			
PL	0.670	0.684	0.643	0.000		
PaP	0.689	0.730	0.682	0.438	0.000	
MP	0.695	0.736	0.630	0.545	0.503	0.000

SP, Batang Berjuntai pheromone (Selangor); SL, Batang Berjuntai light (Selangor); PP, Felcra Berhad pheromone (Perak); PL, Felcra Berhad light (Perak); PaP, Keratong pheromone (Pahang); MP, Indonesia pheromone.

Table 4. Shannon's Information index Lewontin (1972) within six populations of *O. rhinoceros.*

Population	SP	SL	PP	PL	PaP	MP
I	0.3510	0.3547	0.3298	0.3922	0.2835	0.2476
St. Dev	(0.2796)	(0.2873)	(0.2891)	(0.2641)	(0.2792)	(0.2783)

I, Shannon's Information index (Lewontin, (1972)); St. Dev, standard deviation; SP, Batang Berjuntai pheromone (Selangor); SL, Batang Berjuntai light (Selangor); PP, Felcra Berhad pheromone (Perak); PL, Felcra Berhad light (Perak); PaP, Keratong pheromone (Pahang); MP, Indonesia pheromone.

the Pahang Pheromone (PaP) beetles were grouped together as a subcluster. Meanwhile, the Medan Pheromone (MP) samples produced an isolated group in that major cluster.

DISCUSSION

Randomly amplified microsatellite markers are very useful molecular markers that have been widely used to study population genetics, understand polymorphism and identify individual genotypes (Jones et al., 2009 and Wang et al., 2009). RAMs markers have been used widely on different plants and organisms for example in plants like tea; *Camellia sinensis* Latip et al. (2010), rice species Girma et al. (2010) and in insects like plant-hoppers, *Sogatella furcifera* (Liu et al., 2010). This study reports the first use of RAMs markers to assess the genetic variation in *O. rhinoceros*. A high level of polymorphism was revealed in *O. rhinoceros* using the RAMs marker.

In this study, two modes of trapping; pheromone trap and light trap were used to trap *O. rhinoceros* as well as any occurring cryptic species complex that may be attracted to either the pheromone or the light. Using this technique, it was observed that at two locations, Pahang and Medan; beetles were not attracted to light as none was caught in the light trap. Throughout this study, both types of traps were placed along the fringes of replanting sites and the distance between both traps were generally less then 15 m at all study sites. However, at two specific locations in Pahang and Medan, the light trap failed to act as a successful attractant. According to Norman and Basri (2004), *O. rhinoceros* flies about 19 m day-1 and it has the ability to cover a range of 10 to 23 m day-1. Looking at this situation, it could be that *O. rhinoceros* exhibits some level of preference towards light and pheromone thus, increasing the possibilities for the presence of distinct groups.

Generally, species complexes do not interbreed; hence, they are genetically different (Tan, 2006). According to Morehead et al. (2001), cryptic species generally use different chemical signals in mate or gamete recognition but morphological differences among them are difficult to be detected by human observers. During PCR amplification, primers LR5 and VJ1 did not amplify any loci in the Medan Pheromone (MP) population samples. The nucleotide sequences amplified by RAMs primers are the regions between microsatellite loci. If the regions are of suitable length for PCR to take place, then amplification will occur (Jones et al., 2009). Failure of a band to amplify is due to loss of a locus through the deletion of the repeat motifs or primer divergence (Wolfe et al., 1998 and Crawford et al., 2001). In this situation, the failure of LR5 and VJ1 to amplify any products in the Medan Pheromone (MP) population resulted in this population having the lowest percentage of polymorphism (48.72%). It also indicated the uniqueness of the Medan population from the rest.

In this study, 78 polymorphic loci were assessed to understand the population genetic structure of *O. rhinoceros*. The range of value obtained for the Shannon's Information index indicated a low variation within the population. The trapping systems used for

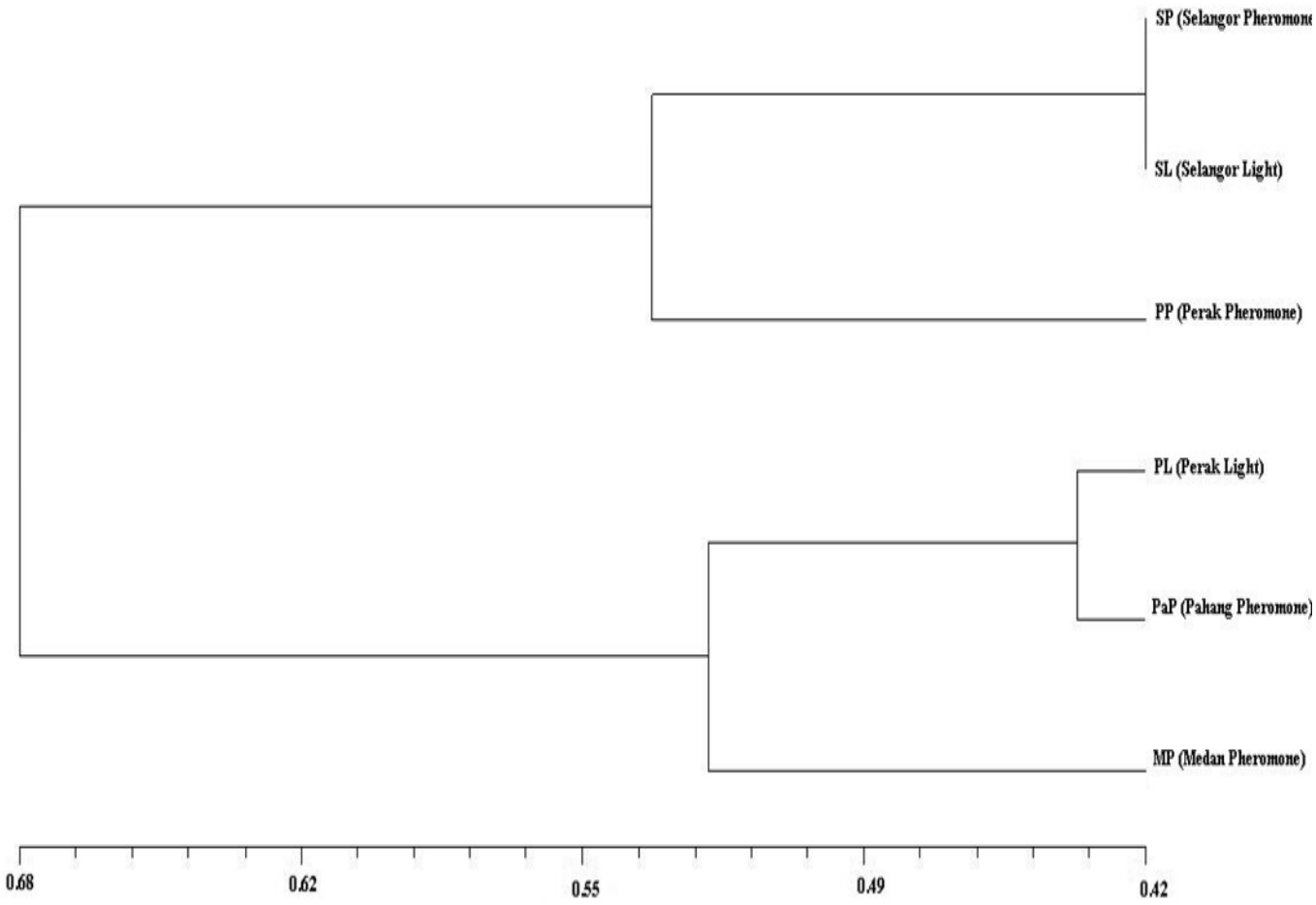

Figure 3. Dendrogram showing the relationships among the six populations of *O. rhinoceros* derived using UPGMA cluster analysis using distances derived from the Nei and Li (1979) similarity coefficients based on RAMs marker data.

population collection as well as the geographical distance were observed to have implications on the dendrogram clustering. The separate clustering of the Perak Pheromone (PP) and the Perak Light (PL) population is an important aspect to be considered. This separation could be possibly due to the presence of two groups of *O. rhinoceros*. It could be assumed that in these study areas, the pheromone trap attracted *O. rhinoceros* which has preference towards pheromone (pheromone group) and also light (light group). Meanwhile, the light trap attracted only *O. rhinoceros* that prefers light (light group). The variation between these two populations was high therefore giving rise to the separation of the Perak Light (PL) population into its own major cluster.

However, the situation in Selangor was different based on the dendrogram. Both the Selangor Pheromone (SP) and the Selangor Light (SL) samples showed the least variation and clustered together. This indicated that, both the different methods of trapping at the location attracted individuals with high similarity among them. It could be possible that all individuals are attracted to light but are also responsive towards the pheromone traps. This showed that, there are no reproductively isolated groups in Selangor. The grouping together of the Selangor Pheromone (SP) and the Selangor Light (SL) may also support the notion that, geographic distance may have specifically contributed to the situation in this area.

In the second major cluster, the Perak Light (PL) and the Pahang Pheromone (PaP) populations grouped as a subcluster. The situation here is similar to the first subcluster, whereby, the Perak Light population would comprise of individual only attracted to light (light group) whereas the Pahang Pheromone (PaP) trap attracted both the pheromone and light preferring groups. However, observing that the Pahang Pheromone (PaP) and the Perak Light (PL) formed a subcluster, this means that the distance between both population were low and most individuals in this subcluster comprised the light attracted groups.

Based on the dendrogram, the Medan Pheromone (MP) population and the Perak Pheromone (PP) population clustered individually as a subcluster in their respective major cluster. Pheromone trap being the major attractant technique at both of the sites may have

attracted a mixture of pheromone and light attracted groups. However, the number of pheromone attracted individuals may be more thus, resulting in them producing an individual subcluster.

Conclusion

In this study, all seven primers that have been used for the screening of the six populations of *O. rhinoceros* have produced highly polymorphic loci. Using these dominant markers, the genetic diversity among *O. rhinoceros* was studied to look into the presence of cryptic species. However, the clustering did not diagnostically group any population to provide a clear evidence of a cryptic complex. Nevertheless, it was possible to understand that the dendrogram clustering did exhibit the possible occurrence of *O. rhinoceros* in two groups which are the pheromone and the light groups. Further studies using codominant markers especially single locus DNA microsatellite markers are required to further understand the population genetic structure of *O. rhinoceros* and to validate the presence of any cryptic species complex. This will in turn contribute towards the efficacy of pest management techniques for rhinoceros beetles in oil palm plantations.

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