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Genetic variation studies in *Oryctes rhinoceros* (L.) (Coleoptera: Scarabaeidae) using single locus DNA microsatellite markers

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Abstract
Oryctes rhinoceros, a serious pest in young oil palm replanting areas, often causes reduced plant productivity and plant death. Common control measures are species specific pheromone traps; however discrepancy in attraction toward the pheromone traps has raised doubts on the possibility of cryptic species complex in *O. rhinoceros.* To ascertain the possibilities of a cryptic species complex; species specific single locus DNA microsatellite markers were used to study the genetic variation within and between *O. rhinoceros* populations. Using 30 species specific single locus DNA microsatellite markers, *O. rhinoceros* beetles from six populations originating from Malaysia and Indonesia revealed no isolated gene pool. Pairwise population differentiation and gene flow values further revealed low to moderate differentiation and a high gene flow between populations. Beetles of different population interacted freely, permitting gene flow between closely and distantly located populations; thus ruling out the possibility for cryptic species complex in *O. rhinoceros*.

Keywords: Aggregation pheromone, Cryptic species complex, Oil Palm, Rhinoceros beetle, Single locus DNA microsatellite markers

1. Introduction
Oil palm is the golden crop of Malaysia. Ever since its introduction in 1911, the oil palm industry has rapidly developed by leaps and bounds resulting in Malaysia being the second highest producer of palm oil in the world after Indonesia [1]. Nevertheless, this industry has its fair share of problems and issues. Infestation of various pests in oil palm plantations is a major issue of concern as failure to manage pest issues may significantly influence the wellbeing of this industry.

Oryctes rhinoceros beetles have been an unremitting dilemma faced by oil palm planters. Commonly known as the rhinoceros beetle, it is an important pest that damages young oil palm plants throughout Malaysia and Indonesia. This beetle has been an incessant agricultural pest since the 18th century. Serious damage to plantations due to *O. rhinoceros* attacks have been well documented throughout. A survey showed that 25% of 180,068 hectare of young palms was attacked by this pest in Malaysia and the beetles were also present in replanting sites as early as six months after replanting [2]. More than 15% reduction in canopy size due to beetle attack had also been observed [3]. This often caused reduction in photosynthetic activity; which further delayed plant maturity, reduced fruit bunch size and caused an approximately 25% crop loss [4]. In addition, an average of 40% crop loss due to beetle attack in the first year of harvesting had also been observed [5].

Basically, suitable environment, abundant food and breeding ground contribute towards the dynamic increase in the number of beetles in plantations. However, the introduction of the "Zero Burning Concept" by the Environmental Quality Clean Air Amendment Regulations 2000 [6] that restricts the burning of palm residues when conducting replanting activities further increased the beetle number. When plantation managements abided by this rule, the infestation of *O. rhinoceros* in plantations became aggravated as there was a drastic increase in the number of available breeding grounds for the beetles in the form of decaying plant materials [7].

Currently, pheromone trapping using a species specific male aggregation pheromone is the most common procedure used to trap and monitor the beetles in young oil palm replanting sites [8]. Although, various control techniques are available, the pheromone has gained popularity among plantation managements due to its efficiency and economical value.

Pheromones are chemical signals from one organism that stimulate a response in another organism of the same species [9]. Male-produced sex attractant have been referred to as aggregation pheromones, because they result in the arrival of both sexes at a calling site leading to an increase in the density of beetles at the pheromone source. Aggregation pheromones are useful for mate selection, defense against predators and overcoming host resistance through mass attack [10]. In *O. rhinoceros* beetles, the aggregation pheromone has also been beneficial for the beetles in finding mates, breeding sites and food [11-12].

However, recently it was observed that not all the population of *O. rhinoceros* in the field was attracted to the pheromone lure (Chung, Ebor Research, Sime Darby Plantations, pers. comm. 2002). Looking at such claims on the discrepancy in attraction toward the species specific pheromone by the beetles, there is a possibility for the occurrence of a cryptic species complex in this insect population. A cryptic species complex is a group of species that are reproductively isolated, hence genetically different [13]. Cryptic species generally use different chemical signals in mate or gamete recognition [14]. Reproductive isolation between populations has been suggested to be an important force in the formation of new species. Prezygotic isolation via behavior isolation such as variation in communication signals like pheromones contribute to reproductive isolation within sympatric species [15]. Therefore, any subtle differences in pheromone components within *O. rhinoceros* beetles could repel selective individuals and thereby contribute to reproductive isolation that leads to the development of a cryptic species complex. Understanding the process of speciation is crucial in pest management. Accurate detection and monitoring of individuals is extremely important, especially when dealing with pest organisms. Generally, the detection of cryptic complex is difficult. This is because cryptic complex often occurs in small population sizes [16]. Failure to identify the presence of reproductively isolated pest species will result in serious errors in pest management in terms of biological control and plantation practice [17].

With interest to understand the *O. rhinoceros* beetles and improvise management and control techniques, many researches have been conducted on this pest development and life cycle [18], habitat [19] and management [20-21]. In addition, a recent study using randomly amplified polymorphic DNA (RAPD) markers [22] and randomly amplified microsatellite markers (RAMs) [23] was carried out on this beetle and both studies had reported the possible presence of two separate gene pools in *O. rhinoceros*. A morphometric analysis on *O. rhinoceros* had also revealed that the beetles are morphologically indistinguishable [24]; consequently strengthening the possibilities for a cryptic species complex within the *O. rhinoceros* species. However, prior to confirmation, analysis using codominant markers such as species specific single locus microsatellite DNA markers is necessary. Over the years, microsatellite markers have become a powerful and promising genetic marker as it allows researchers insights into fine-scale ecological questions.

Therefore, in the effort to understand the genetic variation within and between *O. rhinoceros* populations, as well as to understand the population genetic structure of this pest species and to ascertain the possibilities for the presence of cryptic species complex; a complete population genetic study was carried out on the *O. rhinoceros* populations collected from Malaysia and Indonesia using polymorphic species specific codominant single locus microsatellite DNA markers previously developed [25]. In-depth understanding of this pest species is of high importance for the formulation of future effective pest management programmes. In addition, information obtained from this study will be an added contribution towards the field of entomology.

2. Materials and Methods

2.1 Study locations

This study was conducted at four young oil palm replanting sites whereby three were situated in Peninsular Malaysia and one in Indonesia. The specific study locations were Felcra Berhad in Perak (4^0 8' 46" N; 100^0 50' 22" E), Tennamaram Estate in Selangor (3^0 24' 1" N; 101^0 24' 0" E), Kuantan Trading Plantation in Pahang (2^0 46' 5" N; 102^0 57' 39" E), and Paya Pinang Plantation in Medan, Sumatra (3^0 19' 45" N; 99^0 8' 25" E). The collection of samples was carried out from January 2008 to December 2008. Prior to collection of beetle samples, written permission was obtained from the respective plantation management authorities regarding the use of the plantation for this study. Permission was granted for the setup of traps and also collection of beetle samples throughout the duration of the study.

2.2 Sample collection

At each study site, *O. rhinoceros* samples were collected via two procedures; using the Rothamsted light trap [26] and the pheromone trap using the Sime RB pheromone lures. The light trap which was used in this study uses a lamp producing white light at 200 watts. A collection jar is attached to the light trap structure to collect all attracted beetles. Meanwhile, the pheromone trap is composed of an 18-litre bucket into which a four piece cross sectioned zinc vane is half inserted. The Sime RB pheromone lures is attached to this setup. The trap setting is then hung three to four meters above ground level. The Sime RB Pheromone lures contains ethyl 4-methloctanoate which is a slow-releasing species-specific semiochemical that acts as an aggregation pheromone which attracts both male and female beetles in oil palm plantations [8, 27-28]. Throughout the study, both the light trap and pheromone trap were setup simultaneously at each site for three month duration to collect the beetles. Both traps were stationed along the fringe of replanting sites and the maximum distance between both types of traps was maintained less than 15 meters at all study sites. This distance allows for beetle to have an equal choice in selecting either source of attractant as it is within the flight range of the beetle.

This study incorporates the usage of two different trapping procedures in order to attract both the pheromone responsive and non-responsive individuals. The pheromone trap will attract individuals which are purely responsive towards the pheromone meanwhile, the light trap functions base on positive phototaxis. *O. rhinoceros* are nocturnal organisms that show positive response to phototaxic stimuli [29]. Based on these, we assume that individuals showing differences in attraction towards the pheromone lure, whereby they are not attracted to the Sime RB pheromone lures will be attracted to the light trap. In this manner, the light trap will capture individuals which are less responsive toward the pheromone which could be a possible cryptic complex species that has appeared due to behaviour isolation such as variation in communication signals.

Overall two populations were collected at each study location, namely the pheromone population and the light population. Here after, beetle population are referred based on the location and trapping procedure as in; Selangor Pheromone (SP), Selangor Light (SL), Perak Pheromone (PP), Perak Light (PL), Pahang Pheromone (PaP), Pahang Light (PaL), Medan Pheromone (MP) and Medan Light (ML). To obtain an

accurate representation of the beetles' genetic structure, this study adhered to using 30 individuals per population for analysis as this quantity of samples is suitable when working with a population of unknown level of diversity like the *O. rhinoceros* [30].

2.3 DNA extraction

Genomic deoxyribonucleic acid (DNA) was isolated from beetles using the Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's protocol for animal tissue genomic DNA isolation with minor modifications. The DNA was extracted from the thorax and head tissues of the beetle to avoid contamination of the samples. DNA sample's purity was quantified using a spectrophotometer (Ultrospec III, Pharmacia). The extracted DNA was stored in a -20 ^{0}C freezer.

2.4 Microsatellite genotyping

The 32 polymorphic single locus microsatellite markers developed for *O. rhinoceros* [25] was screened through all the *O. rhinoceros* DNA samples obtained from the six populations. PCR was performed in a 10 µL reaction volume which consisted of 15 ng of genomic DNA, 2.5 mM MgCl2, 1X PCR Buffer, 0.4 mM of each dNTPs, 0.3 µM of each primer and 1U of Taq DNA polymerase (Promega, USA). Amplification was done on a Techne TC-412 thermal cycler with the following cycle profile: 96 ^{0}C initial denaturation for three minutes for one cycle; 95 ^{0}C denaturation for 20 seconds, followed by specifically optimized annealing temperature for 20 seconds, followed by 68 ^{0}C extension for 35 seconds for 35 cycles and a final extension at 68 ^{0}C for five minutes for one cycle.

The PCR products were electrophoresis on 4% Metaphor agarose gel using 1X TBE buffer (0.045 M Tris-borate and 1 mM EDTA, pH 8) at 80 volt for three hours. In addition, a 8% (w/v) vertical polyacrylamide gel electrophoresis (PAGE) using 1X TBE buffer (0.045 M Tris-borate and 1 mM EDTA, pH 8) running at 105 V for one hour was also applied to clearly distinguish band separation. A 20 bp extended molecular marker (Lonza, USA), GeneRulerTM Ultra Low Range DNA Ladder (Fermentas) or 100bp DNA ladder (Fermentas) was used to confirm the product size of each locus. After electrophoresis, the gels were stained in 0.1 µg/µl ethidium bromide and subsequently visualized over UV light and captured using Alpha®Imager 2200 (Alpha Innotech, USA) system.

2.5 Data analysis

The amplified bands were scored using the AlphaEaseFCTM version 4.0 (Alpha Innotech Corporation). Next, the program MICRO-CHECKER [31] was used to identify genotyping errors such as large allele dropout, stutter bands and null allele's presence. Subsequently, CONVERT [32] was used to convert the scored data into input files suitable for subsequent analysis programs. Allele frequencies, observed (N_a) and effective (N_e) number of alleles per locus, polymorphism information content (*PIC*), observed (H_o) and expected (H_e) heterozygosities were calculated and used to evaluate genetic diversity using the software POPGENE version 1.31 [33]. Wright's F statistics (F_{ST}, F_{IS}, and F_{IT}) were estimated using the estimators (θ, f and F) [34]. Meanwhile, the fixation index which estimates F_{IS} as a measure of heterozygote deficiency or excess was also calculated to identify inbreeding within populations [35]. The number of migrants per generation (Nm) which is an indirect estimate of gene flow was also calculated. Allele frequencies at each locus and for each population were tested for conformity to Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium (LD) among all combinations of loci. Bonferroni adjustment was applied to judge the significance level of all simultaneous tests [36]. Bonferroni correction was ($P < 0.05$, minimum adjusted alpha = 0.00028). The above tests were performed using GENEPOP version 4.0.103.4 [37]. In addition, analysis of molecular variance (AMOVA), based on allele frequency information [38] was carried out using Arlequin version 2.0 [39]. The program PowerMarker Version 3.0 [40] was used to generate a tree using the Neighbour Joining algorithm [41] based on the calculated value of D_A distance between population pairs [42]. The robustness of the dendrogram topology was evaluated with a consensus tree generated from 1000 bootstrapping using the program MEGA 5 [43]. Additionally, STRUCTURE [44] was used to infer the actual number of populations based on the microsatellite genotype data.

3. Results and Discussion

3.1 Characteristics of microsatellite loci

This study was initially performed using all the 32 microsatellite loci developed for *O. rhinoceros* [25]. Polymorphic single locus amplifications were observed throughout. However, further analysis of the data set using the Micro-Checker program based on Brookfield null allele estimator 1 [45] indicated that null allele and stuttering occurred in locus OrBPM11-9-1 and locus OrSC3M-5-1. To avoid possible genotyping errors [46] both of these loci were removed from the data set for all the proceeding analysis. The remaining 30 loci were used for analysis and this is sufficient to produce accurate branching pattern for population structure when D_A distance is used [47].

Table 1 highlights the characteristics of all the 30 polymorphic microsatellite loci that were used to screen the six populations of *O. rhinoceros*. Eighty four alleles were successfully amplified within the expected range. The number of alleles in each locus ranged from two to eight alleles and the mean number of allele per locus was 2.8 indicating a low polymorphism. The effective number of alleles per locus ranged between 1.0515 (OrBP11M-8-2) and 4.3835 (OrBP8M-3-4). A large difference between the actual numbers of allele and the effective number of alleles indicates the possible presence of alleles with different frequencies whereby, one allele dominates the allele frequencies and all the others are very rare [48]. This situation was commonly observed in several tested loci. Meanwhile, 18 loci had higher observed heterozygosity (H_o) values compared to the expected heterozygosity (H_e) values. A high heterozygosity at a locus indicates high levels of genetic variation at a locus, which is important as an adaptive response to environmental changes [49]. Overall, the polymorphic information content (*PIC*) for the 30 loci ranged between low (0.047814) to high (0.746129).

Table 1: Characteristics and descriptive statistics of 30 polymorphic single locus microsatellite markers of *O. rhinoceros*

Locus	Primer sequence 5'-3'	Repeat motif	T_a	Observe range(bp)	N_a	N_e	PIC	*Ho*	H_e	GenBank accession
OrBP8M-3-1	F: GGCCTTTCTCGTTGTTGT R: TATAGCCAAGTCGCTGTTCC	$(GTT)_5$ $(AGAAAA)_2$ $(CTGCA)_2$ $(GA)_4$	45.5	256-280	2	1.9302	0.3658	0.5521	0.4834	HM068025
OrBP8M-3-2	F: AGGACTGTGGCGTAATATGG R: TAGTATGGCCGGTAATCTGG	$(AATC)_3$	47.3	308-328	3	2.9313	0.5849	0.7289	0.6608	HM068025
OrBPM8-3-4	F: TTGGATCAATGCTCTGCTTG R: TGGCACCTGGTTGTTGTTG	$(CAA)_6$	49.0	151-187	8	4.3835	0.7461	0.9128	0.7741	HM068025
OrBP11M-1-1	F: ACAATGGAATGACACATACG R: GACGCGAATACCAACAAATC	$(TTAAT)_2$	47.0	163-183	4	1.588	0.3429	0.3184	0.3713	HM068033
OrBP11M-3-1	F: TACAAAGTTTACGGCCAATC R: CGTCCCAATGCCTATTTC	$(AAGAA)_2$	45.3	152-162	2	1.0928	0.0813	0.0889	0.0852	HM068035*
OrBP11M-3-2	F: AAACCATCCGAAAGAAGATG R: TTGTAGATAGGAGACGAGGAAG	$(ACAT)_3$	47.0	170-190	3	1.3111	0.2128	0.2389	0.2380	HM068035*
OrBP11M-7-2	F: CCGGCCATACTATAAAGACG R: TTTGTTTCGGGTAAAGCACAG	$(TA)_3C(TA)_{10}$	47.0	214-248	3	1.3042	0.2203	0.0562	0.2339	HM068037
OrBP11M-8-2	F: CGCAAACCGCACTTTACCAC R: CAACGCTCGTGCTCCTACC	$(TTCAG)_2$	55.0	150-165	2	1.0515	0.0478	0.0503	0.0492	HM068038*
OrBP14M-9-2	F: GCTATCCAGACGGAAAACCT R: CACTACTGATCGTCCTAGTTCTGTT	$(TATTAC)_2$	52.4	115-133	4	1.6185	0.3600	0.4514	0.3833	HM068046*
OrSC3M-4-4	F: TCGTTTCGGCAGTAATATGG R: TGTACGATGAGCGATACTGG	$(TA)_6$	51.5	223-233	2	1.7939	0.3446	0.2768	0.4438	HM068027
OrSC3M-6-3	F: TCGTTGCATGTATGGTTTCG R: GTTGATAATGTCGGTGTTTCTTG	$(ATGT)_4$	48.5	224-240	3	1.1013	0.0897	0.0787	0.0922	HM068029*
OrSC3M-9-2	F: TCGAAGCGAGGAGAATATCG R: CCAGACAGCACAACGTCAAC	$(AG)_4AC(AG)_2$	50.8	281-285	2	1.9164	0.3639	0.7911	0.4797	HM068030
OrVJ2M-1-2	F: CTTTGGTCGTCGCTATTTGC R: GGCTTCACTTGGAACTCCTG	$(TATTA)_2$	49.5	264-274	2	1.218	0.1630	0.1987	0.1795	HM068018*
OrVJ2M-1-3	F: GGTGTTCATGCCTTCCTC R: AAAGATGACCGCCTATTATTG	$(TA)_4$	49.5	197-205	3	2.1706	0.4426	0.75	0.541	HM068018
OrVJ2M-4-2	F: AGCACATATCATCGGGTCAAG R: CGGAAGACTCAAGAAGAAACG	$(AATAAA)_2\ N_7(ATA)_5$	50.8	184-190	2	1.9783	0.3722	0.2558	0.496	HM068019
OrVJ2M-5-3	F: ATTATCCCGTCTGCAAGAAC R: TTAAAGTCCGGGAGCAAGC	$(TAAG)_2$ $(GAAC)_2$ $(TTAA)_2$	49.5	234-242	2	1.1633	0.1305	0.1519	0.1408	HM068020*
OrVJ2M-7-4	F: CTTTTGACCATTGCCTTTGG	$(TAAA)_2(ATTT)_2$	48.2	218-226	2	1.2477	0.1788	0.1765	0.1991	HM068021*

	R: CAGCCCATTTGAATTTTGTG									
OrLR4M-1-2	F: AAAGCAACCGAGTTCGTTC R: GCAATAAACAGATGGCAATG	$(TA)_3N_8(TA)_3N_2(TA)_3$	48.4	242-276	6	3.9200	0.7105	0.5625	0.7470	HM068058
OrLR4M-1-3	F: AAAGTATTTAGCGGATTGCC R: TGGGATTGCCGACTCTTC	$(CGAAA)_2$	46.2	266-276	2	1.1056	0.0909	0.0335	0.0958	HM068058
OrLR4M-2-5	F: TCGAGATAATCAACGTGAGAG R: GGACGCCATTATGTTAAACG	$(AG)_4$	50.2	176-186	2	1.0652	0.0593	0.0632	0.0614	HM068059*
OrLR4M-2-6	F: CTAGTACGCGGTGAGACCTAG R: TGCACATGCAGTGTGTGTG	$(AC)_8$	52.8	113-119	2	1.9912	0.3739	0.9333	0.4992	HM068059
OrLR4M-3-2	F: GCCGAATTGTGAGATGGTTC R: CTTTCAATGTTGCCATCAGC	$(AAACG)_2$	47.5	198-208	2	1.9196	0.3643	0.6784	0.4805	HM068060
OrLR4M-3-3	F: AACCGGAATAAACTCGTCGTC R: CGCATAGTGAGCACAGGAAC	$(AC)_8$ $(GT)_5$ $(TGTAG)_2$	50.2	190-195	2	1.9595	0.3698	0.6601	0.4913	HM068060
OrLR4M-4-5	F: GTAACTAAGCCGGAGGTTCG R: TGCACATGGGAGTGTGTG	$(AC)_8$	52.8	167-171	2	1.9852	0.3731	0.8218	0.4977	HM068061
OrLR4M-6-1	F: GGATGTGCAATGTGTGTG R: CGTTGTCGAACAGTAAACG	$(TG)_7$ $(ATTAA)_2$	48.8	168- 170	2	1.818	0.3487	0.6836	0.4512	HM068063
OrLR4M-7-2	F: CGAACCTCCGGCTTAGTTAC R: AAACCCACAGATCACATTGC	$(AAAAT)_2$	47.0	190-195	2	1.9973	0.3747	0.3504	0.5012	HM068064
OrLR4M-8-2	F: AGGCAGCGGCAGTAAGTG R: TTAAGCCCTCGTTGGACAAG	$(ACGGA)_2$	46.0	199-209	2	1.5207	0.2838	0.4386	0.3434	HM068065
OrLR4M-8-3	F: TGTTACCGTGTTTGATTCATCC R: CAATGTCGAGCCTAGCCAAC	$(T)_{10}$	48.5	217-225	3	1.5271	0.3034	0.2292	0.3464	HM068065
OrLR4M-8-4	F: GTTGGCTAGGCTCGACATTG R: CTAACGACGGGTAGTGATTGG	$(ATTGG)_2$	51.5	244-249	2	1.2079	0.1573	0.0675	0.1726	HM068065
OrLR4M-8-5	F: TTCGCTGACCGACACTTAAC R: ACTGCCATGCCTATCAACAC	$(A)_4T(A)_{10}$	50.5	168-174	6	3.3562	0.6472	0.6875	0.7040	HM068065

Note: T_a – annealing temperature; N_a– number of alleles; N_e – number of effective alleles; *PIC*- polymorphism information content; H_o – observed heterozygosity; *H*e – expected heterozygosity; *P*-value- possibilities to fit to HWE using an exact test. * Deviation from HWE after Bonferroni correction ($P < 0.05$, minimum adjusted alpha = 0.00028).

3.2 Genetic diversity in *O. rhinoceros* beetles

Genetic measures for within population variation for all six *O. rhinoceros* populations using 30 microsatellite markers are shown in **Table 2.** Overall, the total number of allele for all populations ranged between 69 to 76 alleles and the MNE for all the population ranged between 1.7285 and 1.7920. A large difference between the actual numbers of allele and the effective number of alleles was observed within each population; indicating the presence of one allele dominating the frequencies [48]. Overall, allele frequencies varied from one population to another and this could be due to genetic drift, natural selection and human intervention which varied from one area to another [50]. No private alleles that could be used as a diagnostic allele were detected during scoring.

In this study, 57 alleles (67.86%) were shared among all the population. These common alleles were observed to occur at a higher frequency. Common alleles at high frequency indicate the occurrence of selection over generations favouring these alleles. Meanwhile the remaining alleles occurred specifically in particular populations and the frequencies of these alleles were observed to be between 0.0167 and 0.3889. This variation in alleles may have been an important factor that enabled *O. rhinoceros* to adapt and proliferate in an environment that is constantly exposed to various pesticides used to control a wide range of agricultural pest organisms. New alleles appear in a population by the random and natural process of mutation, increasing diversity causing heterozygotes to have the highest fitness leading to it being naturally selected and exhibited in several populations. This therefore favours the maintenance of multiple alleles.

The measure of heterozygosities across all loci is a general indicator of genetic variability and genetic health of a population. A high heterozygosity value, relates to a broader genetic diversity. When comparing among the six populations of *O. rhinoceros*, it was observed that mean observed heterozygosity (H_o) was higher than the mean expected heterozygosity (H_e) in all the populations as shown in **Table 2**. Higher value of observed heterozygosities was indicated in about 15 to 17 loci within each population and this often relates to the polymorphic nature of the locus. In addition, higher observed heterozygosities in a population also highlights the behaviour of the population members that permits the mixing and constant interaction with other populations' members thus contributing to an increase in diversity.

High heterozygosity in a population could be attributed to low level of inbreeding, low selection pressure and large number of alleles present in the population [51]. The situation is further enhanced with the introduction of outbred populations and random mating practises. Based on this, one can assume that the studied *O. rhinoceros* populations are constantly interacting therefore reducing the possibility for the presence of a cryptic species. High heterozygosities will also be indicated when a long term natural selection for adaptation takes place in an organism of mixed nature [52]. Such organism may have historic mixing of strains of different populations. When the process of balancing selection occur [53] heterozygous individuals with naturally higher fitness are selected for survival. In addition, abiotic stress such as pollutants [54-55] has been proven to enhance heterozygote fitness. Factors such as excessive usage of pesticide may also lead to the preferential survival of heterozygotes over homozygotes, indirectly increasing the observed heterozygosities at all the study site. The increase in diversity further indicates the capacity of a population to undergo adaptive evolution to cope with environmental change [56]. Such adaptation within the *O. rhinoceros* could further explain the long term survival of this pest and their capability to adapt to a wide geographical range.

In this study, the average fixation index (F_{IS}) [35] that relates to inbreeding level for each population produced a negative values within -0.1612 (SL population) and -0.0131(PL population) as shown in **Table 2**. The negative values indicated inbreeding does not occur within the population. A higher number in observed heterozygosities in combination with a negative F_{IS} values close to zero signify that individuals were in a random mating state and not under the influence of inbreeding or any other form of assortative mating. Incest avoidance is common in many organisms. However, most species exist in sufficiently large populations that mating between close relatives is unlikely, even in the absence of active incest avoidance mechanisms [57]. This clearly highlights no possibilities for the isolation of gene pools in *O. rhinoceros*.

3.3 HWE and LD in *O. rhinoceros* beetles

The number of loci deviating from HWE for each population ranged within 20 loci (SL population) to 15 loci (PL population) (**Table 2**). Eight loci confirmed to HWE for all the six populations (OrLR4M-2-5, OrVJ2M-7-4, OrVJ2m-1-2, OrSC3M-6-3, OrBP14M-9-2, OrBP11M-8-2, OrBP11M-3-2 and OrBP11M-3-1) and only four loci were found to have deviated from HWE for all the six populations (OrBP8M-3-2, OrLR4M-1-2, OrLR4M-2-6 and OrLR4M-8-5). A total of 75 out of 180 tests (41.11%) conducted conformed to HWE highlighting that a high percentage of the populations are not in HWE. Inbreeding and scoring errors due to null allele and stutter bands interference are not the likely the reasons for the deviation from HWE in *O. rhinoceros* populations. This is because, the negative values of F_{IS} support the non-existence of inbreeding and prior to study, locus with stutter bands and null alleles had been eliminated. Excess of heterozygote individuals, migration and a dominance selection of heterozygote alleles can contribute to deviation from HWE [58] and results obtained on the gene flow and heterozygosity for *O. rhinoceros* population indicated a similar scenario; suggesting it to be the major reason for departures of HWE.

Linkage disequilibrium is the nonrandom association of alleles at different loci within a random mating population [59]. A total of 77 out of 2610 (2.95%) comparisons between all loci by population were found to be significant for LD ($P < 0.05$). Overall, 14 out of 435 (4.58%) loci comparisons showed significant LD across all the six populations. Alleles that indicated the probability of LD were randomly located and no single locus appeared to show LD for its entire alleles. Therefore, alleles were poorly associated within the random mating population. The low level of linkage observed in a randomly mating population indicates that the loci are only statistically linked and not physically linked [60]. Based on the Ohta two locus analysis of population subdivision [61] the total variance (D^2_{IT}) of the disequilibrium was 0.1520. D'^2_{IS} (0.1503) was greater than D'^2_{ST} (0.0017). Meanwhile, D^2_{ST} (0.0389) was greater than D^2_{IS} (0.0033). This revealed that $D^2_{IS} < D^2_{ST}$ and $D'^2_{ST} < D'^2_{IS}$. Therefore observed LD is due to population distance.

Table 2: Genetic measures for within population variation for all six *O. rhinoceros* populations using 30 microsatellite markers

Genetic Measures	Population					
	SP	**SL**	**PP**	**PL**	**PaP**	**MP**
OrBP8M-3-1						
N_a/ N_e	2.0000/1.8672	2.0000/1.9905	2.0000/1.4885	2.0000/1.5463	2.0000/ 1.8127	2.0000/ 1.4593
H_o/ H_e	0.6667/ 0.4723	0.8621/ 0.5064	0.4138/ 0.3339	0.3750/ 0.3608	0.6786/ 0.4565	0.2174/ 0.3217
F_{IS}	-0.4354	-0.7324	-0.2609*	-0.0614*	-0.5135	0.3093*
OrBP8M-3-2						
N_a/ N_e	3.0000/2.7313	3.0000/2.9922	3.0000/2.8771	3.0000/2.5579	3.0000/ 2.6627	3.0000/2.4759
H_o/ H_e	0.6538/0.6463	0.6250/0.6800	0.7143/0.6643	1.0000/0.6201	0.4667/ 0.6350	0.9000/0.6062
F_{IS}	-0.0315	0.0613	-0.0948	-0.6419	0.2527	-0.5098
OrBPM8-3-4						
N_a/ N_e	6.0000/4.3373	6.0000/4.1096	5.0000/2.8526	6.0000/2.5269	7.0000/ 4.9451	7.0000/4.6992
H_o/ H_e	0.9333/0.7825	0.9667/ 0.7695	0.8667/ 0.6605	0.7407 / 0.6157	1.0000/ 0.8113	0.9600/0.8033
F_{IS}	-0.2130	-0.2775	-0.3345	-0.2259*	-0.2535	-0.2195
OrBP11M-1-1						
N_a/ N_e	3.0000/1.7527	3.0000/1.6142	1.0000/1.0000	4.0000/1.8672	2.0000 / 1.4274	4.0000/1.9759
H_o/ H_e	0.1000/0.4367	0.4138/0.3872	0.0000/0.0000	0.5333/0.4723	0.3667 / 0.3045	0.5000/0.5023
F_{IS}	0.7671	-0.0875	-	-0.1483	-0.2245*	-0.0124
OrBP11M-3-1						
N_a/ N_e	1.0000/1.0000	2.0000/1.0339	2.0000/1.0339	2.0000/1.1803	1.0000/ 1.0000	2.0000/1.3423
H_o/ H_e	0.0000/0.0000	0.0333/0.0333	0.0333/0.0333	0.1667/0.1554	0.0000/ 0.0000	0.3000/0.2593
F_{IS}	-	-0.0169	-0.0169	-0.0909*	-	-0.1765*
OrBP11M-3-2						
N_a/ N_e	2.0000/1.1421	2.0000/1.4274	2.0000/1.5139	3.0000/1.7094	1.0000/ 1.0000	3.0000/1.1450
H_o/ H_e	0.1333/0.1266	0.3667/0.3045	0.4333/0.3452	0.3667/0.4220	0.0000 /0.0000	0.1333/0.1288
F_{IS}	-0.0714*	-0.2245*	-0.2766*	0.1165*	-	-0.0526*
OrBP11M-7-2						
N_a/ N_e	3.0000/1.4950	3.0000/1.1102	3.0000 /1.4950	2.0000/1.3423	2.0000/ 1.1421	2.0000/ 1.2277
H_o/ H_e	0.2000/0.3367	0.1034/0.1010	0.0000/0.3367	0.0333/0.2593	0.0000/ 0.1266	0.0000/0.1887
F_{IS}	0.3960	-0.0419*	1.0000	0.869	1.0000	1.0000
OrBP11M-8-2						
N_a/ N_e	1.0000/1.0000	1.0000/1.0000	1.0000/1.0000	2.0000/1.0339	2.0000/ 1.2195	2.0000/1.0713
H_o/ H_e	0.0000/0.0000	0.0000/0.0000	0.0000/0.0000	0.0333/0.0333	0.2000/ 0.1831	0.0690/0.0678
F_{IS}	-	-	-	-0.0169	-0.1111*	-0.0357*
OrBP14M-9-2						
N_a/ N_e	4.0000/1.3339	4.0000/1.8734	4.0000/1.7595	4.0000/2.1264	4.0000/ 1.4207	4.0000/1.2830
H_o/ H_e	0.2759/0.2547	0.6429/0.4747	0.5333/0.4390	0.6897/0.5390	0.3333/ 0.3011	0.2414/0.2244
F_{IS}	-0.1021*	-0.3789*	-0.2355*	-0.3019*	-0.1257*	-0.0943*
OrSC3M-4-4						
N_a/ N_e	2.0000/1.4613	2.0000/1.7076	2.0000/1.3423	2.0000/1.3846	2.0000/ 1.9912	2.0000/1.7241
H_o/ H_e	0.3214/0.3214	0.1724/0.4217	0.1000/0.2593	0.2000/0.2825	0.5333/ 0.5062	0.3333/0.4271
F_{IS}	-0.0182*	0.5839	0.6078	0.2800*	-0.0714*	0.2063*
OrSC3M-6-3						
N_a/ N_e	3.0000/1.1102	2.0000/1.1050	1.0000/1.0000	1.0000/1.0000	2.0000/ 1.0339	2.0000/1.3846
H_o/ H_e	0.0690/0.1010	0.1000/0.0966	0.0000/0.0000	0.0000/0.0000	0.0333/ 0.0333	0.2667/0.2825
F_{IS}	0.3054*	-0.0526*	-	-	-0.0169	0.0400*
OrSC3M-9-2						
N_a/ N_e	2.0000/1.9898	2.0000/1.9179	2.0000/1.8349	2.0000/1.8911	2.0000/ 1.9737	2.0000/1.7817
H_o/ H_e	0.9286/0.5065	0.7931/0.4870	0.7000/0.4627	0.7600/0.4808	0.8846/ 0.5030	0.6500/0.4500
F_{IS}	-0.8667	-0.6571	-0.5385	-0.6129	-0.7931	-0.4815*
OrVJ2M-1-2						
N_a/ N_e	2.0000/1.4706	2.0000/1.2020	2.0000/1.4152	2.0000/1.1050	1.0000/ 1.0000	2.0000/1.0799
H_o/ H_e	0.4000/0.3254	0.1852/0.1712	0.3571/0.2987	0.1000/0.0974	0.0000/ 0.0000	0.0769/0.0754
F_{IS}	-0.2500*	-0.1020*	-0.2174*	-0.0526*	-	-0.0400*
OrVJ2M-1-3						
N_a/ N_e	3.0000/2.1372	3.0000/2.1778	3.0000/1.8893	2.0000/1.8491	2.0000/ 1.8899	3.0000/2.4194
H_o/ H_e	0.7931/0.5414	0.8571/0.5506	0.5625/0.4859	0.5000/0.4675	0.7586/ 0.4791	0.9333/0.5966
F_{IS}	-0.4905	-0.5849	-0.1950*	-0.0889*	-0.6111	-0.5909
OrVJ2M-4-2						
N_a/ N_e	2.0000/1.7241	2.0000/1.6528	2.0000/1.9231	2.0000/1.9802	2.0000/ 1.7875	2.0000/1.0351
H_o/ H_e	0.2000/0.4271	0.2083/0.4034	0.4000/0.4881	0.3000/0.5034	0.3793/ 0.4483	0.0345/0.0345
F_{IS}	0.5238	0.4725	0.1667*	0.3939	0.1390*	-0.0175
OrVJ2M-5-3						
N_a/ N_e	2.0000/1.1128	2.0000/1.0351	2.0000/1.0488	2.0000/1.1389	2.0000/ 1.2800	2.0000/1.3554
H_o/ H_e	0.1071/0.1032	0.0345/0.0345	0.0476/0.0476	0.1304/0.1246	0.2500/ 0.2227	0.3103/0.2668

F_{IS}	-0.0566*	-0.0175	-0.0244	-0.0698*	-0.1429*	-0.1837*
OrVJ2M-7-4						
N_a/ N_e	1.0000/1.0000	1.0000/1.0000	2.0000/1.1590	2.0000/1.0339	2.0000/ 1.4885	2.0000/1.9059
H_o/ H_e	0.0000/0.0000	0.0000/0.0000	0.1481/0.1398	0.0333/0.0333	0.4138/ 0.3339	0.4815/0.4843
F_{IS}	-	-	-0.0800*	-0.0169	-0.2609*	-0.0130*
OrLR4M-1-2						
N_a/ N_e	6.0000/3.6217	6.0000/1.6236	6.0000/4.8613	6.0000/4.3128	5.0000/ 4.2959	5.0000/3.9207
H_o/ H_e	0.5333/0.7362	0.2069/0.3908	0.6897/0.8082	0.5862/0.7816	0.6667/ 0.7802	0.6897/0.7580
F_{IS}	0.2632	0.4613	0.1317	0.2368	0.1311	0.0742
OrLR4M-1-3						
N_a/ N_e	1.0000/1.0000	1.0000/1.0000	2.0000/1.1050	2.0000/1.5139	1.0000/ 1.0000	2.0000/1.0713
H_o/ H_e	0.0000/0.0000	0.0000/0.0000	0.1000/0.0966	0.1000/0.3452	0.0000/ 0.0000	0.0000/0.0678
F_{IS}	-	-	-0.0526*	0.7054	-	1.0000
OrLR4M-2-5						
N_a/ N_e	2.0000/1.0689	2.0000/1.1870	1.0000/1.0000	2.0000/1.1421	1.0000/ 1.0000	1.0000/1.0000
H_o/ H_e	0.0667/0.0655	0.1724/0.1603	0.0000/0.0000	0.1333/0.1266	0.0000/ 0.0000	0.0000/0.0000
F_{IS}	-0.0345*	-0.0943*	-	-0.0714*	-	-
OrLR4M-2-6						
N_a/ N_e	2.0000/2.0000	2.0000/2.0000	2.0000/1.7241	2.0000/2.0000	2.0000/ 2.0000	2.0000/2.0000
H_o/ H_e	1.0000/0.5085	1.0000/0.5085	0.6000/0.4271	1.0000/0.5085	1.0000/ 0.5085	1.0000/0.5085
F_{IS}	-1.0000	-1.0000	-0.4286	-1.0000	-1.0000	-1.0000
OrLR4M-3-2						
N_a/ N_e	2.0000/2.0000	2.0000/1.5158	2.0000/1.9978	2.0000/1.9978	2.0000/ 1.8899	2.0000/1.6653
H_o/ H_e	0.4000/0.5085	0.3478/0.3478	0.9667/0.5079	0.9667/0.5079	0.7586/ 0.4791	0.5517/0.4065
F_{IS}	0.2000*	-0.0222*	-0.9355	-0.9355	-0.6111	-0.3810*
OrLR4M-3-3						
N_a/ N_e	2.0000/1.9976	2.0000/2.0000	2.0000/1.9905	2.0000/1.9413	2.0000/ 1.6897	2.0000/1.6575
H_o/ H_e	0.7586/0.5082	0.9310/0.5088	0.6552/0.5064	0.4783/0.4957	0.4762/ 0.4181	0.5455/0.4059
F_{IS}	-0.5190	-0.8621	-0.3166*	0.0136*	-0.1667*	-0.3750*
OrLR4M-4-5						
N_a/ N_e	2.0000/2.0000	2.0000/2.0000	2.0000/1.9600	2.0000/1.8243	2.0000/ 1.9976	2.0000/1.9802
H_o/ H_e	1.0000/0.5088	1.0000/0.5088	0.8571/0.4987	0.5517/0.4598	0.6207/ 0.5082	0.9000 /0.5034
F_{IS}	-1.0000	-1.0000	-0.7500	-0.2211*	-0.2429*	-0.8182
OrLR4M-6-1						
N_a/ N_e	2.0000/2.0000	2.0000/2.0000	2.0000/1.3423	2.0000/1.4152	2.0000/ 1.5139	2.0000/2.0000
H_o/ H_e	1.0000/0.5088	1.0000/0.5085	0.3000/0.2593	0.3571/0.2987	0.4333/ 0.3452	1.0000/0.5085
F_{IS}	-1.0000	-1.0000	-0.1765*	-0.2174*	-0.2766*	-1.0000
OrLR4M-7-2						
N_a/ N_e	2.0000/1.7875	2.0000/1.7076	2.0000/1.9337	2.0000/1.8491	2.0000/ 1.5414	2.0000/1.4706
H_o/ H_e	0.3793/0.4483	0.5172/0.4217	0.4444/0.4920	0.3333/0.4704	0.0909/ 0.3680	0.1000/0.3282
F_{IS}	0.1390*	-0.2482*	0.0795*	0.2741*	0.7412	0.6875
OrLR4M-8-2						
N_a/ N_e	2.0000/1.4706	2.0000/1.5139	2.0000/1.4922	2.0000/1.3120	2.0000/ 1.1128	2.0000/1.9978
H_o/ H_e	0.4000/0.3254	0.4333/0.3452	0.4167/0.3369	0.2759/0.2420	0.1071/ 0.1032	0.9667/0.5079
F_{IS}	-0.2500*	-0.2766*	-0.2632*	-0.1600*	-0.0566*	-0.9355
OrLR4M-8-3						
N_a/ N_e	3.0000/1.8835	2.0000/1.7423	3.0000/1.5244	2.0000/1.2800	2.0000/ 1.4613	1.0000/1.0000
H_o/ H_e	0.3103/0.4773	0.3846/0.4344	0.3182/0.3520	0.0000/0.2234	0.2500/ 0.3214	0.0000/0.0000
F_{IS}	0.3384*	0.0972*	0.0751*	1.0000	0.2081*	-
OrLR4M-8-4						
N_a/ N_e	1.0000/1.0000	2.0000/1.0713	2.0000/1.3694	2.0000/1.5779	2.0000/ 1.2462	1.0000/1.0000
H_o/ H_e	0.0000/0.0000	0.0000/0.0678	0.1071/0.2747	0.0690/0.3727	0.2222/ 0.2013	0.0000/0.0000
F_{IS}	-	1.0000	0.6028	0.8117	-0.1250*	-
OrLR4M-8-5						
N_a/ N_e	4.0000/3.1690	5.0000/3.5433	5.0000/3.2660	3.0000/2.4663	5.0000/ 2.9801	6.0000/3.6296
H_o/ H_e	0.8000/0.6960	0.6000/0.7299	0.5172/0.7060	0.6897/0.6050	0.6667/ 0.6757	0.8571/0.7377
F_{IS}	-0.1688	0.1641	0.2545	-0.1600	-0.0033	-0.1831
TNA (SD)	73.0000	74.0000	72.0000	74.0000	69.0000	76.0000
MNA (SD)	2.4333(1.2507)	2.4667(1.2521)	2.4000(1.1919)	2.4667(1.1366)	2.3000(1.3170)	2.5333(1.3830)
MNE (SD)	1.7888(0.8013)	1.7285(0.7363)	1.7400(0.8228)	1.7302(0. 6634)	1.7601(0.9217)	1.7920(0.8952)
H_o (SD)	0.4144(0.3529)	0.4319 (0.3576)	0.3761(0.2981)	0.3835(0.3120)	0.3864(0.3164)	0.4339(0.3680)
H_e (SD)	0.3558(0.2393)	0.3451 (0.2270)	0.3420(0.2275)	0.3635(0.2006)	0.3351(0.2372)	0.3484(0.2367)
F_{IS}	-0.1197	-0.1612	-0.0760	-0.0131	-0.1045	-0.1268

*N*a– number of alleles; *N*e – number of effective alleles; *H*o – observed heterozygosity; *H*e – expected heterozygosity; F_{IS} –Inbreeding Coefficient; * - P value of loci conforming to HWE using Fishers exact test with Bonferroni correction (P > 0.05, minimum adjusted alpha = 0.00028). TNA- total number of allele; MNA – Mean number of allele; MNE- Mean number of effective allele, SD – Standard Deviation

3.4 Population differentiation in *O. rhinoceros* beetles

The Wright's F statistics (F_{IS}, F_{IT} and F_{ST}) were calculated based on the estimators (f, F and θ) [34]. The mean value obtained for f and F were - 0.1724 and - 0.0812. Both these negative values indicated there is no inbreeding within the *O. rhinoceros* populations. Meanwhile the θ value of 0.0778 indicates a moderate genetic differentiation among populations; therefore populations are less structured and free to interbreed. As the value is low and close to zero, this implies that the populations are close to panmixia [62]. Meanwhile, the average estimate of gene flow (N_m) was 2.9634; therefore highlighting a high gene flow in the *O. rhinoceros* populations.

The pairwise F_{ST} and N_m values (**Table 3**) between the *O. rhinoceros* populations shows low to moderate differentiation based on the pairwise F_{ST} value which ranged between 0.0218 (SP and SL population) and 0.1482 (PL and MP populations). Meanwhile, the pairwise gene flows observed between the populations (**Table 3**), showed the highest gene flow between SP and SL population (11.2179) and lowest gene flow between MP and PL population (1.4369). Overall, the pairwise F_{ST} values showed significant differences between MP population and PL population only. Other pairwise combinations showed no significant differences.

Table 3: Pairwise F_{ST} and N_m value for all six populations of *O. rhinoceros*

	SP	SL	PP	PL	PaP	MP
SP	****	11.2179	4.6138	4.7500	3.8686	1.6269
SL	0.0218	****	1.6269	3.8217	3.1057	1.5869
PP	0.0514	0.1332	****	9.7500	5.0466	1.5434
PL	0.0500	0.0614	0.0250	****	4.2303	1.4369
PaP	0.0607	0.0745	0.0472	0.0558	****	3.0308
MP	0.1332	0.1361	0.1394	0.1482*	0.0762	****

Note: Diagonally above – Pairwise gene flow (*Nm*) values; Diagonal below – Pairwise F_{ST} value; *: P-value for F_{ST} after 1000 permutation. Nominal adjusted level (5%) for multiple comparison = 0.003333; *: P< 0.003333 = significantly different from zero

Looking at the results obtained the lowest F_{ST} value was between Selangor Pheromone (SP) population and Selangor light (SL) population. This particular pair also generated the largest N_m value. The locations of both the populations were the major reason behind the generated value. Both populations being trapped from the same state in the same plantation resulted in them sharing a same gene pool. This is clearly highlighted by the low F_{ST} value which indicates the lack of differences in the population structure between both the sets. The high gene flows between both this populations indicate transfer of alleles between both populations. This situation hereby suggests that although both populations were trapped using different procedures, there however seemed to be no specific isolation of gene pool between them. In addition, no diagnostic alleles were also found to prove an occurrence of specific differences. When we look at the F_{ST} and N_m value between Perak Light (PL) and Perak Pheromone (PP) population, conditions similar to that occurring between Selangor Light (SL) and Selangor Pheromone (SP) population were observed. Similarity in location between Perak Light (PL) and Perak Pheromone (PP) populations may have been the reason for a high gene flow and low F_{ST} value between this pairs. This is clearly highlighted by the low F_{ST} value which indicates the lack of differences in the population structure between both the sets. The population pair that exhibited the highest F_{ST} value (0.1482) and the lowest N_m (1.4639) value was between Perak Light (PL) and Medan Pheromone (MP) population. The Straits of Malacca Sea could have acted as a geographical barrier between both this sites therefore resulting in the lowest gene flow between this population pair. However, it must be highlighted that although the observed N_m value for this population pair was comparatively the lowest, it was still insufficient to highlight any major restricted gene flow that could result in an isolated population.

An N_m value more than 0.5 indicates that an organisms is capable of dispersing its genes over a large geographic distances [63]. When observing the N_m values calculated in this study, is could be confirmed that the beetles of different population interacted freely, thus permitting gene flow between closely and distantly located populations. A lack of differentiation between the population and a high gene flow between all the populations was observed. This has proved that populations collected via different trapping procedures did not show any form of genetic isolation. Therefore the attraction level to both light and pheromone were in par for all beetles regardless of location. This also proves that *O. rhinoceros* has the ability to disperse its genes over a large geographic distances therefore possessing a higher degree of similarity. This unites the organism into a homogenous genetic group that evolve together [64]. Interpopulation gene flow within species reduces population differentiation thus when genes are exchanged between two populations, speciation is prevented by the recombination of genes which are associated to reproductive isolation and adaptations to different niches [63]. This in turn acts against speciation thus reducing any possible chances for the occurrence of cryptic species complex within the *O. rhinoceros* beetles.

The AMOVA analysis (**Table 4)** further confirmed that there exists no cryptic species complex in *O. rhinoceros*. Majority of variation occurred within populations (92%), meanwhile among populations variation was accountable for only 8%. The high intrapopulation variability and genetic homogeneity across populations could have arisen due to high levels of gene flow and random mating between population [65] and this further leads to a genetically homogenous group of individuals in *O. rhinoceros*

Table 4: Summary of Analysis of Molecular Variance

Source of Variation	df	Sum of Squares	Mean Squares	Variance of Components	Variance (%)
Among Populations	5	159.1060	31.821	0.443	8
Within Populations	354	1849.6110	5.225	5.225	92
Total	359	2008.717	37.046	5.668	100

3.5 Population structure of *O. rhinoceros* beetles

Genetic distance measure (D_A) [42] was calculated for all the six populations of *O. rhinoceros* as in **Table 5**. The highest distance was between PL and MP population with a value of 0.0742. Meanwhile the smallest genetic distance was between SP and SL population with a value of 0.0174. A neighbour joining tree (**Figure 1**) constructed for the six *O. rhinoceros* populations based on the distance measures of D_A and further confirmed with a consensus tree using 1000 set bootstrapping, highlights three major clusters. The first major cluster grouped was PaP and MP population (91 % of clustering). Meanwhile, the second major cluster grouped SL and SP population

together (95% of clustering). The third major cluster grouped PP and PL population together (87% of clustering).

Table 5: Genetic distance D_A among six populations of *O. rhinoceros*

	SP	SL	PP	PL	PaP	MP
SP	0.0000					
SL	0.0174	0.0000				
PP	0.0366	0.0383	0.0000			
PL	0.0397	0.0348	0.0243	0.0000		
PaP	0.0424	0.0386	0.0397	0.0360	0.0000	
MP	0.0628	0.0651	0.0723	0.0742	0.0445	0.0000

Note: SP-Selangor Pheromone; SL-Selangor Light, PP- Perak Pheromone; PL- Perak Light; PaP- Pahang Pheromone; MP- Medan Pheromone

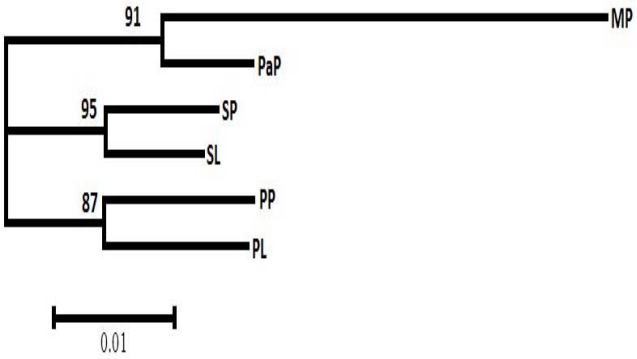

Fig 1: Neighbour joining tree generated from genetic distance measure (D_A) between six populations of *O. rhinoceros* based on 30 polymorphic single locus microsatellite loci.
Note: SP-Selangor Pheromone; SL-Selangor Light, PP- Perak Pheromone; PL- Perak Light; PaP- Pahang Pheromone; MP- Medan Pheromone

Based on cluster two (SP and SL population) and cluster three (PP and PL population), it could be suggest that similarity in location is the reason for the clustering pattern. It was revealed that populations which were trapped using two different methods from the same location were actually corresponding to the same gene pool indicating very low genetic differentiation between them. This situation is further supported by the high N_m values which indicate consistent gene flow occurring between the pairs of populations from the same locations. Occurrence of random mating in these populations as highlighted previously by F_{IS} values and low genetic structuring as proved by the F_{ST} value further relates to the reason for the clustering pattern. Based on these two clusters, it could be confirmed that the hypothesis on possible presence of a cryptic species complex in *O. rhinoceros* that is indicated by the selective attraction towards pheromone trapping due to possible variations in chemical signalling can be ruled out.

Meanwhile, cluster one in (PaP and MP population) further proved that the occurrence of gene flow between the populations as a strong reason for the low genetic differentiation between the population. The clustering of these two populations is interesting as both populations originate from different countries and are geographically isolated from each other by the Straits of Malacca which is more than 500 nautical miles long and ranges between a width of 126 nautical mile (233.35 km) at the northern region to approximately 9 nautical miles (16.67 km) at the southern region between Peninsular Malaysia and the Indonesian island; Sumatera [66]. Similarities in gene pool between organisms separated by geographical barrier like sea is common and have been reported in other species too [67-68]. Many species are shared between the islands on the Sunda Shelf due to their prehistoric connected land platform during Pleistocene that supported dispersal of species. Further incidence of climatic oscillations also supports species distributions by promoting the dispersal to new locations or migration out and later returning to proliferate [69-70]. In addition, the beetles have a flight range of the 19 m day-1 or more than 130 m per week in a replanting site that is equipped with abundance of food and breeding sites [12]. Earlier reports have suggested that under dire state and in need of food and breeding ground, this pest has the ability to fly for a considerably long distance [71-72]. There are various records on the flight range of these beetles for example; a flight distance of 700m had been recorded in Mauritius [73] and also laboratory experiment that had indicated that the beetle can fly up to two to four kilometres in two to three hours [72]. Reports on flight distance of 140 m into replanting area had also been recorded [4]. Therefore, when influenced by natural instinct to breed and search for food, the flight capability of this beetle does support distant migration. Apart from that, shipping, cargo transportation, nursery trade and transportation of habitat material could have also facilitated the migration and lack of differentiation between populations of *O. rhinoceros* as seen in the clustering [18].

Meanwhile, to infer population structure based on Bayesian technique using the STRUCTURE software, the admixture model based on correlated allele frequencies was used to test the data set. Initial analysis was carried out using a burning length and MCMC run of 10,000 to 100,000 at an iteration of 20 for K=2 to K=7. A finalized burning length and MCMC of 100,000 was selected when values of summary statistics like α and *LnP (D)* appeared to have converged. Three population clusters was obtained based on the delta *K* value. The delta *K* value or likelihood for the number of clusters was $K = 3$ at 35.90. **Figure 2** shows the bar plot observed using the Bayesian clustering method from the program STRUCTURE. The proportion of membership of each predefined population in each of the three clusters is shown in **Table 6**. Based on the proportion of membership in each cluster, cluster one grouped PP and PL population, cluster two grouped SP and SL population and cluster three grouped PaP and MP population. Although three major cluster can be observed, individuals still had some association with other cluster as mixing was observed in the population assignment. None of the predefined populations had a 100% membership in the newly assigned clusters which is a clear indication of the presence of gene flow among all populations. This again highlights no presence of isolated gene pool that indicates a cryptic species complex.

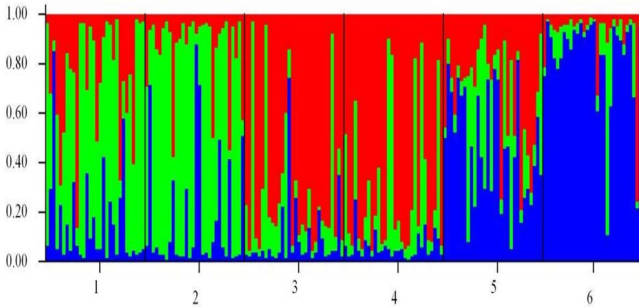

Fig 2: Bar plots showing Bayesian clustering patterns of the genotype data assuming admixture and correlated allele frequency when K=3.
Note: Number of burnings: 100,000; MCMC: 100,000; Iterations: 20; Population identities: (1) Selangor Pheromone (2) Selangor Light (3) Perak Pheromone (4) Perak Light (5) Pahang Pheromone and (6) Medan Pheromone; Cluster: 1-red, 2-green, 3-blue.

Table 6: Proportion of membership of each predefined population in each of the three clusters based on Bayesian analysis.

Population	Cluster 1	Cluster 2	Cluster 3	No of Individuals
SP	0.249	0.587	0.164	30
SL	0.134	0.686	0.180	30
PP	0.710	0.195	0.095	30
PL	0.708	0.228	0.064	30
PaP	0.307	0.220	0.474	30
MP	0.079	0.094	0.827	30

Note: SP-Selangor Pheromone; SL-Selangor Light, PP- Perak Pheromone; PL- Perak Light; PaP- Pahang Pheromone; MP-Medan Pheromone; Cluster: 1-red, 2-green, 3-blue

4. Conclusion

A detailed analysis on the population genetic variation within and between *O. rhinoceros* populations using species specific single locus microsatellite markers has revealed a lack of differentiation between the *O. rhinoceros* populations. Presence of high gene flow between populations resulted in no isolated gene pools. This clearly rules out possibilities for the presence of cryptic species complex in *O. rhinoceros*. This study has proved that the selective attraction as claimed to be exhibited by the beetles toward the pheromone trapping system is not due to prezygotic isolation behaviour commonly exhibited by cryptic species of sympatric nature. This study has successfully confirmed the population genetic structure of *O. rhinoceros* and information obtained could be utilized in future pest management programmes.

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