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A preliminary investigation of genetic diversity amongst Rusa timorensis in Tanjung Malim, Perak and Bilut Agro Farm, Pahang, Malaysia

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Abstract

A study on genetic diversity analysis was conducted on *Rusa timorensis* obtained from state of Perak and state of Pahang to investigate the level of genetic diversity occur and to compare the diversity amongst two *R. timorensis* breeders in Malaysia. A total of six (n=6) individual samples of *R. timorensis* were extracted from Tanjung Malim, Perak and Bilut Agro Farm, Pahang and amplified using mitochondria deoxyribonucleic acid (mtDNA) primers gene as a target molecular marker. The mtDNA region was amplified using a set of cytochrome b gene primers (5″AAACCA GAAAAGGAGAGCAAC3″;5″TCATCTAGGCATTTTCAGTGCC3″) and nucleotide sequence of the mtDNA cyt b was aligned by using MEGA Ver 7.0. The result indicated that the *R. timorensis* from Pahang has a low degree of variation (0.252) of genetic distance compared to, *R. timorensis* from Perak (0.696). The phylogenetic three analysis, indicated, *R. timorensis* from Pahang resulted the highest intra-specific relationship at 100% compared to, *R. timorensis* from Perak at 63% of intra-specific relationship. The results showed that the genetic diversity of, *R. timorensis* in Perak and Pahang is likely to decrease in the future. Therefore, future breeding program plan needs to be implemented to diversify the genetics of genus Rusa in Malaysia.

1. INTRODUCTION

Rusa timorensis are the largest deer species and they are medium-sized deer, rough-coated deer and anthropogenic activities of *R. timorensis* influenced by habitat, human hunting activities and translocation (Ali et al., 2021). The criteria that effect the antropogenic activities of this deer species lead to substantial impact on genetic diversity and the long-term conservation of local population of this species.

The *R. timorensis* natively occurs on the islands of Java, Bali, and Timor in Indonesia. It has been introduced to Irian Jaya, Borneo, the Lesser Sunda Islands, Maluku, Sulawesi, Pohnpei, Mauritius, Reunion, Fiji, Tonga, Samoa, Vanuatu, the Solomon Islands, the Christmas Islands, the Cocos Islands, Nauru, Australia, New Caledonia, New Zealand, Papua New Guinea, New Britain, and New Ireland (Hedges et al., 2015; Long, 2003; Sutrisno, 1993).

Most breeder in Malaysia reared deer from *R. timorensis* because this species is easily adapted with the current environment and resistant to disease (Abdul Kadir, 2003). Therefore, *R. timorensis* have a potential for meat supply demand and hunting purposes legally or illegally and because of these major activities *R. timorensis* population is reportedly likely to decline, therefore it needs to be observed systematically and continuously, so that the potential of this biological resource can be developed optimally (Zein and Saim, 2001).

Furthermore, the breeder facing problems to sustain the population of this species because the supply demand too high. In order to overcome this situation, efficient management of wild or captive deer population with the aim to maintain the deer population viable and enhance potential breeding performance of farmed population requires the development of molecular genetic tools (Bonnet et al., 2002; Zein and Maharadatunkamsi, 2003).

This study have used a sequence of nucleotide sequence fragments of mitochondrial DNA control area to provide a clear picture of the phylogeography of deer populations in Perak and Pahang. The approaches of mtDNA that are well known as specific, reproducible, sensitive, rapid processing time and low costs has adapted in the molecular technique with the intention to obtain the DNA sequence and construct the intra-specific relationship for these six *R. timorensis*. Specifically, mitochondrial

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DNA has proven to be an ideal sequence for phylogeographic analyses due to its high rate of sequence evolution, uniparental inheritance, and lack of recombination and also variable marker for phylogenetic genetic diversity purposes (Avise, 2000; Lowe et al., 2004).

Thus, this paper presents the results of the genetic diversity analysis of the *R. timorensis* population in Perak and Pahang for the sustainability of this species and to evaluate the genetic consequences of the history of the *R. timorensis* introduction into Malaysia. As a further action, the potential detection of successive bottlenecks with the initial introduction of deer and subsequent establishments of deer farms, current farming practices regarding breeding programmes and the spatial distribution and reproductive patterns of farmed deer populations.

2. MATERIALS AND METHODS

2.1. Sampling and DNA extraction

A total of six (n=6) individual genomic samples of *R. timorensis* were taken from in Perak (3 individuals) and Pahang (3 individuals) in February 2020. A total of 10g meat of *R. timorensis* was taken from each individual and frozen immediately until DNA extraction with MasterPure™ Complete DNA and RNA Purification Kit from Biosearch Technologies, United Kingdom. The extraction protocol was followed according to the manufacturer protocol. The DNA were then kept in -4°C before performing optical density reading to measure the purity of obtained DNA.

2.2. Partial mtDNA cytochrome b gene sequencing

The final DNA concentrations in the 25 µL PCR reaction mixture were: 12.5 µL of PCR master mix (1.5 unit Taq polymerase (Promega), 10 mM Tris-HCL, 50 mM KCL, 1.5 µM dNTPs, and 1.5 mM MgCl2 Promega 10 X PCR reaction buffer), 2 µL of DNA, 8 µL of water, and 1 µL of pM each of the forward and reverse primers (Helix BioTech). Amplification of DNA product was successfully performed using the specific primer pair (F- 5”AAAC CAGAAAAGGAGAGCAAC3”) and (R- 5”TCATCTAG GCATTTTCAGTGCC3”) for *R. timorensis.*

The PCR amplification was carried out in a Biotherm (Analytik Jena, GmBH). The thermal cycler program was conducted of an initial pre-denaturation step at 95°C for 5 minutes, followed by 40 cycles that consisted of a denaturing step at 95°C for 30 seconds, an annealing step at 60°C for 30 seconds, and an extension step at 72°C for 40 seconds. This step was followed by a final extension step for 10 minutes at 72°C and stored at a constant 4°C C in the refrigerator. The PCR products were analysed by horizontal electrophoresis on a 1.5% (w/v) agarose gel in 1 X TBE Buffer at 120 volts for 1 hour.

The amplicon was cleaned using GeneAll Purification Kit (GeneAll Biotechnology Co, LTD, Kr). The fragment was sequenced automatically in both directions by using a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystem, USA). The cycle sequence reaction was performed in a Thermal Cycler (Takara Bio USA, Inc) with the following parameters: 35 cycles of 10 seconds at 96°C, 5 seconds at 50°C, and 4 minutes at 60°C. The PCR cycle sequencing product was then purified using a DyeX Purification kit (QIAGEN, Gmbh) and the fragment was then sequenced using an automatic sequencer ABI 3500 Genetic Analyzer (Applied Biosystem, USA) (Begum et al., 2019).

2.3. DNA analysis

The DNA sequences were analysed using software program Molecular Evolution Genetic Analysis (MEGA) version 7.0 (USA) (Kumar et al., 1993) to compute and to obtain the consensus sequence of *R. timorensis* meat DNA sequence by multiple alignments of the forward and reverse sequences. Then, the DNA sequences of the *R. timorensis* meat were blasted with the National Center For Biotechnology Information (NCBI) to ascertain the identity of the *R. timorensis* based on the consensus of DNA sequence obtained. The genetic distance of this species was then computed using p-distance calculation to obtain the genetic variation and intra-specific relationship between these two *R. timorensis* in different location. Then from the genetic distance obtained, phylogenetic tree was constructed using bootstrapping method.

3. RESULTS AND DISCUSSION

3.1. Detection of *R. timorensis* DNA

F DNA is crucial in any polymerase chain reaction (PCR) analysis and without DNA, PCR process lacks of results and sequencing as well (Abdul Kadir, 2003). The DNA quality is important prior to PCR analysis. The DNA extracted product was successfully amplified and identified as *R. timorensis* by using a cyt b gene primer set. As shown in Figure 1.0, the DNA showed sharp band on 1.5% (w/v) agarose gel. All deer samples were positive with deer DNA, indicated a single band with a molecular weight of 358 bp and the finding confirmed that the DNA extracted from deer meat was a deer DNA using the oligonucleotide primers.

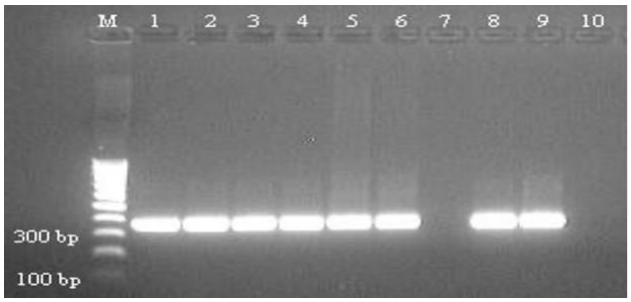

Figure 1: Amplicon of *R. timorensis* DNA isolated from raw meat. Lane M: 100 bp ladder; Lane 1-3; *R. timorensis* (Tanjung Malim, Perak); Lane 4-6: *R. timorensis* (Bilut, Pahang); Lane 7: Blank; Lane 8: PCR Control (Chicken); Lane 9: Extraction Control (Chicken); Lane 10: Water.

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3.2 Genetic distance variation

Preliminary study by using 358 bp cyt b primer, approximately 313 nucleotide sequence of *R. timorensis* corresponding to loci 14,273 – 14,586 of the entire *R. timorensis* mtDNA retrieved from the GenBlast database (accession number MF 279249.1, Martin et al., 2017). In total, 6 meat DNA samples were individually tested in the PCR and sequencing assays. Using these assays, the study conducted molecularly authenticated the species of deer for all meat sample taken 3 from Tanjung Malim, Perak and 3 from Bilut, Pahang (Figure 1). Using both assays, the results obtained showed specifically for 6 meat samples there are genetic variation occurred between deer from Perak and Pahang (Figure 2)(Table 1).

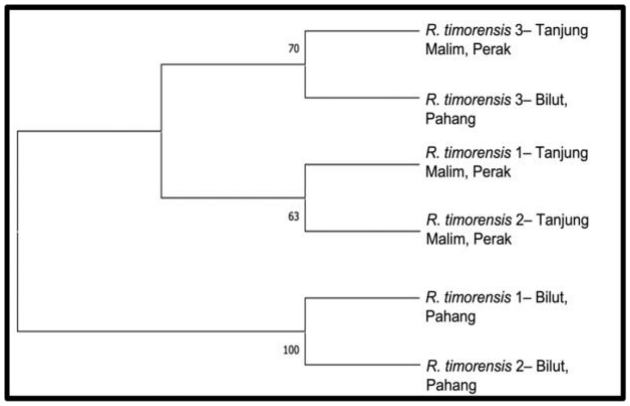

Figure 2: Phylogenetic tree of relationship among six (6) different individual of *R. timorensis* collected from Tanjung Malim, Perak and Bilut, Pahang.

The result indicated *R. timorensis* 1 Bilut, Pahang and *R. timorensis* 2 Bilut, Pahang to display a fairly low degree of genetic distance (0.252) among other *R. timorensis.* The phylogenetic tree analysis constructed for *R. timorensis* individual 1 and 2 from Pahang showed high of intraspecific relationship at 100%. While *R. timorensis* 3 Bilut, Pahang and *R. timorensis* 3 Tanjung Malim, Perak surprisingly showed low degree of genetic distance (0.686), but showed high of intraspecific relationship at 70% in contrast with *R. timorensis* 1 Tanjung Malim, Perak and *R. timorensis* 2 Tanjung Malim, Perak with 63% of intraspecific relationship at 0.696 genetic distance.

Similarly, the study conducted by Zein (2007) is parallel with this study which low genetic variation observed between *R. timorensis* in Indonesia islands. With the high variability observed in *R. timorensis* in the state Pahang, with proper management, parentage determination and relatedness, there is potential for breeding stock establishment compared to Perak population. This due to the value of 100% intraspecific- relationship observed in Pahang deer population and it can be an used as an effective tool for accurately examining population processes such as dispersal patterns and population structuring in *R. timorensis* species in order to solve the population bottleneck (Webley et al., 2004). In contrast with *R. timorensis* (Perak) which showed low genetic variability at 63% of intraspecific relation. This preliminary study is alarming the breeders possibly increase the hybrid of *R. timorensis* farming with other *R. timorensis* which have high variability like Pahang species and its required co-operation from all breeders in Malaysia to provide the deer meat to be analysed for better impact.

This could also be further explained as a consequence of sampling too few informative characters or due to this species yet to become monophyletic for their respective mitochondrial lineages as a result of geographical separation. Therefore, the application of PCR technique in this study indicates high bootstrap support for the mtDNA region to be used in discriminating between the species. This high support for the conspecific grouping of *R. timorensis* species based on the phylogenetic p-distance analysis illustrates the potential of mDNA gene to be used in intraspecific differentiation.

Table 1: Genetic distance comparison data for *R. timorensis* samples from Tanjung Malim, Perak and Bilut, Pahang compute using p-distance formula.

	***R. timorensis*-3-Tanjung Malim, Perak**	***R. timorensis*-1-Bilut, Pahang**	***R. timorensis*-2-Bilut, Pahang**	***R. timorensis*-3-Bilut, Pahang**	***R. timorensis*-1-Tanjung Malim, Perak**	***R. timorensis*-2-Tanjung Malim, Perak**
***R. timorensis*-3-Tanjung Malim, Perak**						
***R. timorensis*-1-Bilut, Pahang**	0.761					
***R. timorensis*-2-Bilut, Pahang**	0.742	0.252				
***R. timorensis*-3-Bilut-Pahang**	0.686	0.742	0.761			
***R. timorensis*-1-Tanjung Malim, Perak**	0.745	0.776	0.788	0.736		

4. CONCLUSION

Study has shown the genetic diversity among *R. timorensis* between two location. Those breed was potentially used in improvements in breeding and selection programmes of farmed *R. timorensis* species. Furthermore, the preliminary data indicated the farmed *R. timorensis* from Pahang and Perak were significantly different in term of genetic distance and intra-specific relationship even though it's from same family and this factor maybe be due to geographical condition and thorough studies need to be conducted specifically on geographical differences factor.

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