POPULATION GENETIC STRUCTURE OF MALAYAN TAPIR (Tapirus indicus Desmarest) IN PENINSULAR MALAYSIA

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By

LIM QI LUAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

POPULATION GENETIC STRUCTURE OF MALAYAN TAPIR (*Tapirus indicus* Desmarest) IN PENINSULAR MALAYSIA

By

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March 2019

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The Malayan tapir (*Tapirus indicus* Desmarest) is an endangered fauna listed in the International Union for Conservation of Nature (IUCN) Red List with estimated population size at less than 2,000 individuals in Peninsular Malaysia. Despite the existing conservation programme and ecological information about this species, the population genetic structure of the Malayan tapir in Peninsular Malaysia is still not well-known, largely due to a lack of available genetic markers. The lack of such information may impede the on-going efforts for its conservation and management. The works presented here aimed to develop genetic markers for the investigation of population genetic structure of the Malayan tapir in Peninsular Malaysia. Forty-one microsatellite markers comprising of seven random amplified microsatellite (RAM)-isolated and 34 cross-amplification microsatellite markers, obtained from literature and National Center for Biotechnology Information (NCBI) database, were screened with polymerase chain reaction (PCR), sequencing and fragment analysis in 67 Malayan tapirs. Eight polymorphic markers were successfully developed and used in the population genetic structure analysis. Using K-means clustering algorithm, five clusters were inferred among the wild samples (N = 57), which showed a complex population structure probably comprising multiple continuous populations that also experiencing considerably restricted gene flow due to isolation by geographical barriers especially mountain ranges. Mitochondrial control region sequences in Peninsular Malaysia samples (N = 44; including two samples from Singapore Zoo) revealed two clades that might be established during the late Pleistocene. One of the clades was exclusive in Peninsular Malaysia samples in comparison with the Thailand samples from a previous study. However, the geographical distribution of the clades did not show a clear population structure. A total of 12 novel haplotypes were detected. Both the markers suggested low to moderate genetic diversity in the Malayan tapir studied. In addition, a universal sex-typing method based on the sex-

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determining region Y and zinc finger gene (as positive control) was tested. A preliminary assessment of sex ratio was conducted using the data extracted from the tapir datasheets obtained from the Department of Wildlife and National Parks, Sungai Dusun Wildlife Conservation Centre and Zoo Negara; and aided with the developed sex-typing marker for those biological samples with unknown sex. Overall, there was no significant bias towards either sex. Nevertheless, in the wild-born tapirs, the sex ratio seemed to favour females and the opposite was observed in the captive-born tapirs. From 2004 to 2015, there seemed to be an increase in the male proportion but no extreme ratio was found. Combined with microsatellite data, there was no sex-biased dispersal detected in a spatial autocorrelation analysis that might shape the population structure of the Malayan tapir observed. A major limitation in all these studies was the sampling bias where, across Peninsular Malaysia, more samples were sampled from the Selangor-Negeri Sembilan-Pahang regions and only a few were representatives of the populations from the north forest complexes.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

STRUCKTUR POPULASI GENETIK TAPIR MALAYA (*Tapirus indicus* Desmarest) DALAM SEMENANJUNG MALAYSIA

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Tapir Malaya (*Tapirus indicus* Desmarest) adalah sejenis haiwan terancam yang tersenarai dalam Senarai Merah Kesatuan Antarabangsa untuk Pemuliharaan Alam Sekitar (IUCN) dengan saiz populasi dianggar kurang daripada 2,000 ekor di Semenanjung Malaysia. Walaupun terdapat program pemuliharaan dan maklumat ekologi tentang spesies ini, struktur genetik populasi Tapir Malaya di Semenanjung Malaysia masih belum dikaji sepenuhnya, sebahagian besarnya disebabkan oleh kekurangan penanda genetik yang sedia ada. Kekurangan maklumat sedemikian boleh menghalang usaha berterusan untuk pemuliharaan dan pengurusannya. Kajian ini bertujuan untuk menguji penanda-penanda genetik dan seterusnya memakainya untuk mengkaji struktur genetik populasi Tapir Malaya di Semenanjung Malaysia. Empat puluh satu penanda mikrosatelit sedia ada di literatur dan data *National Center for Biotechnology Information (NCBI)* yang terdiri daripada tujuh penanda RAM (*Random Amplified Microsatellite*) dan 34 penanda mikrosatelit yang diperoleh daripada spesies tapir lain telah diuji dengan reaksi berantai polimerase (PCR), penjujukan dan analisis genotip pada 67 Tapir Malaya. Lapan penanda polimorfik berjaya dikembangkan dan digunakan dalam analisis struktur genetik populasi tapir. Kaedah gugusan K-means telah mencadang kewujudan lima kluster genetik Tapir Malaya di antara populasi tapir liar (N = 57). Taburan kluster-kluster tersebut di Semenanjung Malaysia turut menunjukkan struktur populasi yang kompleks dan berkemungkinan terdiri daripada beberapa populasi berterusan yang juga mengalami aliran gen terhad mungkin disebabkan oleh faktor pengasingan geografi seperti banjaran gunung. Analisis terhadap jujukan nukleotida *control region* mitokondria dalam sampel Semenanjung Malaysia (N = 44 termasuk dua sampel daripada Zoo Singapura) telah mencadang kewujudan dua klad pada zaman Pleistosen Akhir. Salah satu daripada klad tersebut adalah eksklusif dalam sampel Semenanjung Malaysia berbanding dengan sampel dari Thailand dalam kajian

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terdahulu. Walau bagaimanapun, pertaburan geografi klad tidak menunjukkan struktur populasi yang jelas. Sejumlah 12 haplotip baharu telah diperoleh. Kedua-dua penanda genetik mencadangkan kepelbagaian genetik yang rendah atau sederhana dalam populasi Tapir Malaya yang dikaji. Di samping itu, kaedah mengenal pasti jantina berdasarkan gen penentuan seks pada kromosom Y dan gen "*zinc finger*" (sebagai kawalan positif) telah diuji. Penilaian awal nisbah jantina dilakukan menggunakan data yang diperoleh daripada Jabatan Hidupan Liar dan Taman Negara, Pusat Konservasi Hidupan Liar Sungai Dusun dan Zoo Negara, dan dibantu dengan kaedah mengenal pasti jantina tersebut untuk sampel biologi yang tidak diketahui jantina. Secara keseluruhannya, nisbah tapir jantan dan betina tidak berbeza secara signifikan. Walau bagaimanapun, dalam populasi tapir liar, nisbah jantina nampaknya memihak kepada betina dan keadaan sebaliknya didapati dalam populasi tapir yang dilahirkan dalam kurungan. Dari tahun 2004 hingga 2015, peningkatan kadar jantan didapati walaupun perbezaan nisbah jantan dan betina adalah tidak ketara. Apabila data mikrosatelit diuji mengikut kategori seks, tidak ada perbezaan didapati dalam taburan genetik antara tapir jantan dan betina yang mungkin membentuk struktur populasi Tapir Malaya. Satu kelemahan utama dalam semua kajian ini adalah pensampelan yang kurang merata di rantau Semenanjung Malaysia. Iaitu lebih banyak sampel telah diperoleh dari kawasan Selangor-Negeri Sembilan-Pahang tetapi hanya segelintir sampel diperoleh dari kawasan utara Semenanjung Malaysia yang dirangkumi kompleks hutan yang luas.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

+G	(including) Gamma distribution
+I	(including) Invariant site
λ	Number of substitutions per site per year
π	Nucleotide diversity
2n	Diploid number
3’	End of a linear DNA strand
5’	Beginning of a linear DNA strand
6-FAM	6-carboxyfluorescein (a fluorescent dye for fragment analysis)
A	Adenine
AMOVA	Analysis of Molecular Variance
AU	Action unit
BIC	Bayesian Information Criterion
bp	Base pair
C	Cytosine
CLUMPAK	Cluster Markov Packager Across K
CR	Control region
CSB	Conserved sequence block
CytB	Cytochrome *b*
delta *G*	Gibbs energy
DAPC	Discriminant analysis of principal component
d_N	Number of substitutions per site
DNA	Deoxyribonucleic acid
DBS	Dried blood spot
PERHILITAN	Department of Wildlife and National Parks
ESU	Evolutionarily significant unit
ETAS	Extended termination associated sequences
F	Fixation index
Fst	Fixation index (inbreeding coefficient)
G	Guanine
GADM	Database of Global Administrative Areas
gDNA	Genomic DNA
H_	Name prefix for haplotype
Hd	Haplotype diversity
H_e	Expected heterozygosity
HKY	Hasegawa, Kishino & Yano model
H_o	Observed heterozygosity
HEX	A type of fluorescent dye for fragment analysis
HWE	Hardy-Weinberg equilibrium
I	Shannon’s information index
IBD	Isolation-by-distance
IUCN	International Union for Conservation of Nature
K	Number of genetic clusters
K-means	Unsupervised learning algorithm for clustering observations
K80	Kimura 2-parameter model
LD	Linkage disequilibrium
LIZ500	Dye-labelled size standard with 16 fragments up to 500 bp

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MaxEnt	Maximum entropy
MCMC	Markov chain Monte Carlo
MLG	Multilocus genotype
MJ	Median-joining
ML	Maximum likelihood
MP	Multiplex panel
mtDNA	Mitochondrial deoxyribonucleic acid
MU	Management unit
MYA	Million years ago
N	Count or number
N_a	Allele number
N_e	Effective allele number
NCBI	National Center for Biotechnology Information
NJ	Neighbour-joining
Nm	Number of effective migrants per generation
PC	Principal component
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PIC	Polymorphic information content
PM	Peninsular Malaysia
RAM	Random amplified microsatellite
RNA	Ribonucleic acid
ROX	A type of fluorescent dye for fragment analysis
SD	Standard deviation
SNP	Single nucleotide polymorphism
SRY	Sex-determining region Y
SSR	Simple sequence repeat
STR	Short tandem repeat
T	Thymine
Taq	*Thermus aquaticus*
t_D	Divergence time
TH	Thailand mtDNA CR dataset
T_a	Annealing temperature
T_m	Melting temperature
uH_e	Unbiased heterozygosity
UTR	Untranslated region
WCC	Wildlife Conservation Centre
WGRB	Wildlife Genetic Resource Bank
X	X chromosome
Y	Y chromosome
ZF	Zinc finger
ZFX	X-linked zinc finger gene
ZFY	Y-linked zinc finger gene

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CHAPTER 1

INTRODUCTION

Genetic variation, which lays the basis for genetic diversity and thus genetic structure, is one of the three levels of biodiversity i.e. genetic diversity, species diversity and ecosystem diversity, that deserve to be conserved. Genetic diversity is often correlated to population fitness and surviving capacity of the population through adaptation (Barrett & Schluter, 2008; Reed & Frankham, 2003). International Union for Conservation of Nature (IUCN) ranking of a species as ‘Vulnerable’, ‘Endangered’, or ‘Critically Endangered’ is based on its statistics of population decline and range loss, which in turn implies a significant extinction risk faced by the concerned species as well as suggests a reduced genetic diversity by inbreeding and drift in the threatened populations (Rivers, Brummitt, Lughadha, & Meagher, 2014; Willoughby et al., 2015).

The Malayan tapir (*Tapirus indicus* Desmarest) is one of the endangered faunas listed in the IUCN Red List (Traeholt et al., 2016). The Malayan tapir is found in Southeast Asia including Thailand, Sumatra and Peninsular Malaysia. Its population in Peninsular Malaysia is declining due to threats such as habitat loss, habitat fragmentation and road kills. In Malaysia, the setting up of the Malayan Tapir Conservation Centre and the development of the Malayan Tapir Action Plan are among the efforts to conserve this species. The conservation efforts include captive breeding management and operations such as the rescue of displaced or injured tapirs, and reintroduction or translocation of the tapirs from one population to another (Magintan, Traeholt, & Karuppanannan, 2012; Mahathir et al., 2014).

While habitat protection and maintenance, and at one point, conservation intervention by humans are important to maintain the Malayan tapir population, population genetic diversity should not be overlooked in the conservation biology of the Malayan tapir. Examination of the amount of genetic diversity and its distribution pattern over a geographic area can provide valuable insights into the population genetic structure—number of subpopulations, genetic variation within the subpopulations, and the degree of gene flow between them, as well as make inference to the factors and demographic processes that shaped the genetic structure of the population (Allendorf, Luikart, & Aitken, 2013; Chakraborty, 1993). Understanding the Malayan tapir population genetic structure has important management and conservation implications for the species, for example, identifying population management units that may be genetically distinct from each other will help authorities such as Department of Wildlife and National Parks (PERHILITAN) in Malaysia to take caution when making decisions on reintroduction, translocation and breeding. Furthermore, the genetic data and information can be used for long-term monitoring programme for both wild and captive Malayan tapir populations. Prior information on population genetic structure will also allow wildlife

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conservationist to design their experiment or research plan for testing more sophisticated hypotheses using subpopulations as groups.

Population studies of the Malayan tapir based on conventional methods such as camera trapping and radiometry (e.g. Rayan et al., 2012; Traeholt & Sanusi, 2009; K. D. Williams, 1979) to study its population distribution, population density, home range etc. did not include the information on population genetic structure, which requires molecular or genetic markers to explore. The genetic approach offers advantages in term of grasping population information at the molecular level that is inaccessible by conventional methods, yet with proper research design and assessments, it can reveal similar population information obtained from the latter techniques and even more. For example, while both approaches can be used to estimate population size and density (Janečka et al., 2011), population structure, in the sense of distribution of individuals in a geographical area, can be more readily assessed by evaluating distribution pattern of their genetic diversity using samples from various sources, rather than employing ecological field techniques to track down movement of a number of individuals. Genetic markers such as nuclear microsatellite markers (Pinho, Gonçalves da Silva, Hrbek, Venticinque, & Farias, 2014) and mitochondrial deoxyribonucleic acid (mtDNA) markers (Muangkram, Amano, et al., 2016) are popular tools for estimating genetic diversity within or among subpopulations.

Estimation of sex ratio and sex-biased dispersal, which are among the factors that influence population genetic structure, can be achieved with sex-identification markers e.g. sex-determining region Y (SRY) and zinc finger (ZF) gene for identifying sexes of collected samples (Pelizzon, da Silva Carvalho, Caballero, Manoel Galetti Junior, & Sanches, 2017; Quaglietta, Fonseca, Hájková, Mira, & Boitani, 2013). However, studies with these markers i.e. microsatellite, mtDNA, and sex-identification markers are still few or largely lacking in the Malayan tapir, if not totally absent, especially for populations residing in Peninsular Malaysia and Sumatra. Only a few genetic research on the Malayan tapir population, whether captive or wild, were conducted in the past decade. These projects focused mainly on the mtDNA genes e.g. cytochrome *b* (CytB) gene and control region (CR) to assess genetic diversity, phylogenetic or phylogeographic relationships in Thailand (Muangkram, Amano, et al., 2016; Muangkram et al., 2013) and Japan zoos (Ogata, Watanabe, & Ogawa, 2009), and in Peninsular Malaysia (Rovie-Ryan et al., 2008). Others include research that only aimed to reconstruct phylogenetic relationships among members of Tapiridae (Ashley, Norman, & Stross, 1996; de Thoisy et al., 2010).

As such, more genetic markers need to be developed to lay the ground for further studies in the population structure and diversity for the Malayan tapir, as well as using the genetic information for improving *in-situ* and *ex-situ* conservation management. Therefore, the aim of this thesis and the research works performed within it was to fill the gap in the knowledge by developing and using genetic markers to provide novel insights into the population genetic

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structure of the Malayan tapir in Peninsular Malaysia, to account for the current situation where the population genetic structure of Malayan tapir has remained not well-understood despite its ‘Endangered’ status. The objectives were:

1. to screen and characterise 41 microsatellite markers in the Malayan tapir, which includes seven novel microsatellite marker loci isolated from the Malayan tapir by random amplified microsatellite (RAM) markers and 34 microsatellite marker loci developed for the lowland tapir, Baird’s tapir, and mountain tapir;

2. to assess the genetic diversity and population genetic structure of the Malayan tapir in Peninsular Malaysia using the tested polymorphic microsatellite markers;

3. to assess the genetic diversity, population genetic structure and phylogenetic relationships of the Malayan tapir population in Peninsular Malaysia using mtDNA CR and with the inclusion of Thailand captive samples.

4. to verify and characterise the SRY/ZF sexing method in Malayan tapir for sex-typing samples of unknown sex, which are to be included for a preliminary assessment of sex ratio in the wild and captive-born Malayan tapir populations in Peninsular Malaysia, and to detect spatially sex-biased dispersal in relation to microsatellite data and geographical distances in the wild population.

Chapter 1, as has been described in this chapter, introduces the main research subject of this project—the Malayan tapir, clarifies the problems faced by the fauna and the main aim of this thesis in contributing valuable genetic tools and information for the conservation of the Malayan tapir. Chapter 2 gives a review of the subjects relevant to this project. Chapter 3 describes the development of microsatellite markers and its use to assess and clarify the population genetic structure of the Malayan tapir in Peninsular Malaysia. While Chapter 3 investigates population genetic structure in the Malayan tapir using biparentally inherited microsatellite markers, Chapter 4 investigates the population genetic structure using maternally inherited mtDNA CR. Other than population structure, the chapter also investigates phylogenetics and genetic diversity of Malayan tapir in Peninsular Malaysia by including the mtDNA CR sequences of the Malayan tapir kept in Thailand. Chapter 5 describes the development of a sex-typing method for samples of unknown sex. The sex data was then used to estimate sex ratio in the wild- and captive-born Malayan tapirs in Peninsular Malaysia. In addition, data from microsatellite markers and the sex data were combined to look for population structure caused by the differential in dispersal in different sexes. Chapter 6 provides a general discussion on the results obtained through Chapter 3 to Chapter 5. Lastly, Chapter 7 gives a recap of all the works conducted for objectives in Chapter 3 through Chapter 5 and the conclusions made and provides recommendations on what can be researched in the future to widen the knowledge about the ecology and genetics of the Malayan tapir.

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