UNIVERSITI PUTRA MALAYSIA

MOLECULAR GENETIC CHARACTERIZATION OF RUSA (*CERVUS TIMORENSIS*) AND SIKA (*CERVUS NIPON*) DEER SPECIES IN MALAYSIA

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By

KOUROSH JOME KHALEDI

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

April 2008

DEDICATION

TO YOU

MY FATHER AND MOTHER,

MY WIFE AND MY DAUGHTER,

AND ALSO TO

PROFESSOR DR. MOSTAFA CHAMRAN

WHO WAS A REAL SCIENTIST AND RELIGIOUS MAN

AND FINALY WAS MARTYRIZED IN THE WAR

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

MOLECULAR GENETIC CHARACTERIZATION OF RUSA (*CERVUS TIMORENSIS*) AND SIKA (*CERVUS NIPON*) DEER SPECIES IN MALAYSIA

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April 2008

Chairman: Associate Professor Jothi Malar Panandam, PhD

Faculty: Agriculture

The Malaysian livestock industry is an important component of the agricultural sector providing gainful employment and producing useful animal protein food to the population. Cattle, buffalo, goat, sheep and swine are the popular livestock in Malaysia. However, in recent years deer farming for meat, fur and velvet has become popular as well. Most of the farmed deer are of various species imported from different sources, such as Australia, New Zealand, Mauritius, Indonesia, New Caledonia, etc. The genetic background of these species and populations from different sources are unknown. This study was conducted to characterize two popular deer species in Malaysia, namely the rusa deer (*Cervus timorensis*) and the sika deer (*Cervus nippon*), using DNA microsatellite markers.

The use of amelogenin gene primers for sexing of the rusa deer was also investigated. Random samples of 38 rusa deer from the Deer Breeding Unit of

the University Research Park, Universiti Putra Malaysia, and 34 sika deer from Pusat Ternakan Haiwan, Batu Arang, were used in the study to determine and compare the genetic structures of the two deer species.

One hundred and twenty five sets of microsatellite primer pairs, which had been reported to have successfully detected variation in deer, cattle or sheep, were used in the initial screening. Thirty nine primer pairs produced clear and reproducible amplification products for rusa and 41 primer pairs for sika. Twenty one primer pairs were polymorphic for the pooled data. However, only nine microsatellite loci (23.08%) were polymorphic for rusa and 17 loci (41.46%) were polymorphic for sika. Of these, only five were common to both deer species (BMS789, BM888, BL4, BM3628 and NVHRT16). Of the monomorphic loci, 17 were common to both species. Among the 11 reindeer microsatellite loci screened, nine loci (81%) were amplified for the pooled data, but only four loci were common to both species. The two white-tailed deer microsatellite loci (L35582 and L35583) produced amplification in sika but only L35583 was amplified in rusa. The 17 common monomorphic loci and the nine polymorphic loci generated in total 53 and 40 microsatellite markers in the rusa and sika, respectively. Locus BM2113 was amplified exclusively in rusa (126 bp), and locus NVHRT34 was amplified only in sika (134 bp). These loci may be used as unique markers to distinguish the two deer species.

The numbers of observed and effective alleles per polymorphic loci were 2 - 13 and 1.05 - 8.91 for rusa, and 2 - 8 and 1.16 - 5.98 alleles per locus for sika, respectively. The allele frequencies ranged from 0.01 to 0.97 for rusa, and 0.02

to 0.92 for sika. The sizes of the alleles at the polymorphic loci ranged from 116 to 389 bp in rusa, and 88 to 364 bp in sika. The mean numbers of effective alleles were 3.08±2.40 and 2.87±1.65 in rusa and sika, respectively. Six loci in rusa and three loci in sika exhibited rare alleles. The mean observed heterozygosity in the rusa and sika populations were 0.48 ± 0.35 and 0.51 ± 0.30, respectively. Seven polymorphic loci in rusa and 14 polymorphic loci in sika exhibited significant ($P<0.01$) deviations from Hardy-Weinberg equilibrium. The Hardy-Weinberg disequilibrium may be due to overlapping of generations and founder effect, especially in the sika deer population. The combined discrimination power (cDP) of the nine polymorphic loci in rusa was 0.99 and of the seventeen polymorphic loci in sika was 0.99, thus allowing individual identification. The inbreeding coefficient (F_{IS}) was very low for the rusa population (0.06), but for the sika population it was 0.26. The mean value of F_{ST} was 0.67 for rusa and 0.47 for sika. The bottleneck analysis suggested that the rusa population did not experience any recent bottleneck, whereas the sika population had encountered a genetic bottleneck in the recent past. Evaluation of intra-interchromosomal linkage disequilibrium between the alleles suggested significant ($P<0.05$) linkage between 10 pairs of alleles in rusa and 12 pairs of alleles in sika. However, none of the allelic pairs were the same for the two species. The genetic distance within the rusa population was lower than that within the sika population (0.088 ± 0.001 vs. 0.184 ± 0.001). The genetic distance between rusa and sika was 0.35. No distinct clustering was observed for the rusa population. The sika population displayed two major clusters of 11 and 23 individuals. The larger cluster in turn had two sub-clusters.

The above results show the rusa and sika populations to be genetically different from each other. High genetic variation exists in both the populations. This could be due to low inbreeding and no directional selection in these populations.

Four amelogenin gene primer pairs were used to identify the sexes of the rusa deer. Three primer pairs, $AMEL_2$, AMGX/Y and $AMGX/Y_2$, exhibited similar banding patterns for the males and females. The primer pair SE47/48 generated one band for the females (269 bp) but three bands (223, 269 and 305 bp) for the males. Therefore, this primer pair is a reliable tool for the identification of the sexes in the rusa deer.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PENCIRIAN GENETIK MOLEKUL SPESIS
RUSA (*CERVUS TIMORENSIS*) DAN SIKA (*CERVUS NIPON*)
DI MALAYSIA**

Oleh

KOUROSH JOME KHALEDI

April 2008

Pengerusi : Profesor Madya Jothi Malar Panandam, PhD

Fakulti : Pertanian

Industri peternakan di Malaysia adalah komponen penting dalam sektor pertanian dan menyediakan peluang pekerjaan dan sumber makanan protein haiwan kepada populasi. Lembu, kerbau, kambing, biri-biri dan babi adalah ternakan yang poular di Malaysia. Walau bagaimana pun, pada beberapa tahun kini peternakan rusa untuk daging, bulu dan velvet juga menjadi popular. Kebanyakan rusa yang diternak adalah pelbagai spesis dan diimport dari sumber berlainan, seperti Australia, New Zealand, Mauritius, Indonesia, New Caleonia dan sebagainya. Latar belakang genetik spesis dan populasi tersebut adalah tidak diketahui. Kajian ini telah dijalankan untuk mencirikan dua spesis rusa yang popular di Malaysia, iaitu rusa (*Cervus timorensis*) dan sika (*Cervus nippon*), dengan menggunakan penanda DNA mikrosatelit.

Penggunaan primer gen amelogenin untuk pengelasan jantina rusa turut disiasat. Sampel rawak 38 ekor rusa dari Unit Pembiakbakaan Rusa, Taman Penyelidikan Universiti, Universiti Putra Malaysia, dan 34 ekor sika dari Pusat

Ternakan Haiwan, Batu Arang, telah digunakan dalam kajian ini untuk menentukan dan membandingkan struktur genetik kedua-dua spesis rusa.

Sejumlah satu ratus dua puluh lima set pasangan mikrosatelit, yang telah dilaporkan sebagai berjaya mengesan variasi dalam rusa, lembu, kambing atau biri-biri, telah digunakan dalam pengujian awalan. Tiga puluh sembilan pasangan primer menghasilkan produk amplifikasi yang jelas dan boleh dihasilkan semula untuk rusa dan 41 pasangan primer untuk sika. Dua puluh pasangan primer adalah polimorfik bagi data terkumpul. Walaubagaimanapun, hanya sembilan (23.08%) lokus mikrosatelit adalah polimorfik untuk rusa dan 17 lokus (41.46%) adalah polimorfik untuk sika. Antara ini, hanya lima adalah sama untuk kedua-dua spesis rusa (BM5789, BM888, BL4, BM3628 dan NVHKT16). Antara lokus monomorfik, 17 adalah sama bagi kedua-dua spesis. Antara 11 lokus mikosatelit reindeer yang dikaji, sembilan lokus (81%) adalah diamplifikasi bagi data terkumpul, tetapi hanya empat lokus adalah sama bagi kedua-dua spesis. Dua lokus mikosatelit rusa white-tail (L35582 dan L35583) hasilkan amplifikasi pada sika tetapi hanya L35583 diamplifikasi dalam rusa. Tujuh belas lokus monomorfik yang sepunya dan sembilan lokus polimorfik telah menghasilkan sejumlah 53 dan 40 penanda mikrosatelit dalam rusa dan sika, masing-masing. Lokus BM2113 diamplifikasi khusus dalam rusa (126 bp), dan lokus NVHR734 diamplifikass hanya dalam sika (134bp). Lokus ini boleh digunakan sebagai penanda unik bagi membezakan kedua-dua spesis rusa tersebut.

Bilangan alel yang dicerap dan efektif per lokus polimorfik adalah 2 - 13 dan 1.05 - 8.91 untuk rusa, dan 2 - 8 dan 1.16 - 5.98 alel per lokus untuk sika,

masing-masing. Frekuensi alel berjulat antara 0.01 ke 0.97 bagi rusa, dan 0.02 ke 0.92 bagi sika. Saiz alel pada lokus polimorfik berjulat antara 16 ke 389 bp dalam rusa, dan 88 ke 364 bp dalam sika. Purata bilangan alel yang efektif adalah 3.08± 2.90 dan 2.87±1.65 dalam rusa dan sika, masing-masing. Enam lokus dalam rusa dan tiga dalam sika menunjukkan alel yang jarang. Purata heterozigositi yang dicerap dalam populasi rusa dan sika adalah 0.48 ± 0.35 dan 0.51 ± 0.30, masing-masing. Tujuh lokus polimorfik dalam rusa dan 14 lokus polimorfik dalam sika menunjukkan sisihan signifikan ($p<0.01$) daripada keseimbangan Hardy-Weinberg. Ketidakseimbangan Hardy-Weinberg mungkin adalah kerana pertindihan generasi dan kesan penubuh, terutamanya bagi populasi sika. Kuasa diskriminasi bersatu (cDp) sembilan lokus polimorfik dalam rusa adalah 0.99 dan bagi 17 lokus polimorfik dalam rusa adalah 0.99, maka membenarkan pengenalpastian individu. Koefisyen pembiakbakaan dalam (F_{IS}) adalah sangat rendah bagi populasi rusa (0.06), tetapi bagi populasi sika ia adalah 0.26. Nilai purata F_{st} adalah 0.67 bagi rusa dan 0.47 bagi sika. Analisis 'bottleneck' mencadangkan populasi rusa tidak mengalami masalah 'bottleneck' yang baru-baru ini, manakala populasi sika telah mengalami 'bottleneck' genetik pada masa baru-baru ini. Penilaian ketidakseimbangan 'intra-interchromosomal linkage' di antara alel mencadangkan rangkaian signifikan ($p<0.05$) di antara 10 pasangan alel dalam rusa dan 12 pasangan alel dalam sika. Walau bagaimanapun, tiada sebarang pasangan alel yang sama untuk kedua-dua spesis. Jarak genetik dalam populasi rusa adalah lebih rendah berbanding dengan yang dalam populasi sika (0.088±0.001 melawan 0.184±0.001). Jarak genetik di antara rusa dan sika adalah 0.35. Tiada kluster yang yang diperhatikan untuk populasi rusa.

Populasi sika menunjukkan dua kluster major dengan 11 dan 23 individu. Kluster yang lebih besar mempunyai dua sub-kluster

Keputusan di atas menunjukkan populasi rusa dan sika adalah berbeza dari segi genetik. Variasi genetik yang besar wujud dalam kedua-dua populasi. Ini mungkin disebabkan oleh pembiakbakaan dalam yang rendah dan tiada pemilihan berhala dalam populasi tersebut.

Empat pasangan primer gen amelogenin telah digunakan untuk mengenalpasti jantina rusa. Tiga pasangan primer, $AMEL_2$, AMGX/Y dan $AMGX/Y_2$, menunjukkan kesamaan corak 'banding' bagi jantan dan betina. Pasangan primer SE47/48 menghasilkan satu Jalurn untuk betina (269 bp) tetapi tiga jalur (223,269 dan 305 bp) untuk jantan. Maka, pasangan primer ini adalah alat yang sesuai untuk mengenalpasti jantina rusa.

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I certify that an Examination Committee has met on 11th April 2008 to conduct the final examination of Kourosh. J. Khaledi on his Doctor of Philosophy thesis entitled ''Molecular Genetic Characterization of Rusa (*Cervus timorensis*) and Sika (*Cervus nipon*) Deer Species in Malaysia'' in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the degree of Doctor of Philosophy.

Members of the Examination Committee are as follows:

Abdul Razak Alimon, PhD
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Faculty of Agriculture
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Tan Soon Guan, PhD
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Date: 26 May 2008

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

Jothi Malar Panandam, PhD
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Siti Shapor Siraj, PhD
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Professor and Dean
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Date: 12 June 2008

DECLARATION

I declare that the thesis is my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or other institution.

KOUROSH JOME KHALEDI

Date: 26 June 2008

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LIST OF ABBREVIATIONS

A	Adenine
AE	Elution buffer
AFLP	Amplified Fragment Length Polymorphism
AL	Lysis buffer
AMELX	amelogenin gene in chromosome X
AMELY	amelogenin gene in chromosome Y
AMG	amelogenin gene
AMOVA	analyses of molecular variance
AW1	Wash buffer 1
AW2	Wash buffer 2
bp	Base pair
C	Cytosine
cDP	combined discrimination power
CSB	clone sequence based
dNTP	dinucleotide triphosphate
DP	Discrimination Power
DVS	Department of Veterinary Services
EDTA	ethylene diamine tetra acetic Acid
EPA	Extreme Preferential Amplification
EtBr	ethidium bromide
F	Fixation Index
G	Guanine
GATA4	gata binding protein 4
H_o	Observed heterozygosity
H_e	Expected heterozygosity
HMG	high mobility group
HWE	Hardy-Weinberg equilibrium
IAM	Infinite Allele Model
LD	linkage disequilibrium
MAS	marker assisted selection
$MgCl_2$	magnesium chloride
ml	Milliliter
mM	Millimolar
mRNA	messenger ribonucleic acid

MSA	Microsatellite Analyser
mtDNA	mitochondrial DNA
Na	observed number of alleles
Ne	effective number of alleles
ng	nanogram
P	Panmictic Index
PAGE	Polyacriramid Gele Electrophoresis
PCR	Polymerase Chain Reaction
PIC	Polymorphism Information Content
QTL	Quantitative Trait Loci
RAHM	Random Amplified Hybridization Microsatellite
RAMPO	Random Amplified Microsatellite Polymorphism
RAPD	Random Amplification of Polymorphic DNA,
RFLP	Restriction Fragment Length Polymorphism
rpm	rotation per minute
SF1	Steroidogenic Factor 1
SMM	Stepwise Mutation Model
SNP	Single Nucleotide Polymorphism
SOX9	Sry-related HMG box gene 9
SRX	Sex Determination Region in chromosome X
SRY	Sex Determination Region in chromosome Y
SSR	Simple Sequence Repeats
STR	Short Tandem Repeats
T	Thymine
TBE	Tris borate ethylene diamine tetra acetic acid
TDF	Testis Determination Factor
T_m	Melting temperature
TPM	Two-Phase Model
TSPY	Testis- Specific Protein Y-encoded
U/µL	unit per microlitter
UPGMA	Unweighted Pair Group Method with Arithmetic mean
UPM	Universiti Putra Malaysia
UV	ultraviolet
VNTRs	variable number of tandem repeats
WT1	Wilm's Tumor gene 1
ZFX	Zinc Finger protein in chromosome X

ZFY	Zinc Finger protein in chromosome Y
µl	microliter
µM	micromolar
^{0}C	Centigrade Celsius
1X	one time