AMPLIFICATION OF ZINC FINGER PROTEIN GENE (ZFX) AS IDENTIFICATION MARKER FOR MALAYSIAN BOVINE SPECIES

by

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CERTIFICATE

This is to certify that the dissertation entitled "Amplification of Zinc Finger Protein Gene (zfx) as Identification Marker for Malaysian Bovine Species" is the bonafide record of research work done by Mohd Hafiz Mail during the period from July 2008 to October 2008 under my supervision.

Date:
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OBJECTIVE OF STUDY

The objective of this study is to amplify the zinc finger protein gene from bovine genomic DNA derived from leucocytes by polymerase chain reaction (PCR). The zfx gene then is sequenced to analyze the specific enzyme restriction site using computer software. Identification of bovine species can be diagnosed through restriction fragment polymorphism.

The establishment of a molecular genetic marker for cattle could help us to support the conservation or breeding plans as well as an enforcement tools for the identification law for threatened species in Malaysia.

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ABBREVIATION

IUCN	International Union for Conservation of Nature
Zfx	bovine zinc finger protein gene (X chromosome derived)
Zfy	bovine zinc finger protein gene (Y chromosome derived)
R & D	Research and Development
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
ACD	Anticoagulant-citrate-dextrose
EDTA	Ethylenediaminetetraacetic
SDS	Sodium dodecyl sulphate
MW	Molecular weight
PBS	Phosphate buffer saline
TE	Tris-EDTA
µL	microliter
µg	microgram
mM	milimolar
mL	mililiter
°C	degree centigrade
ng	nanogram
nM	nanomolar

TBE	Tris-Borate-EDTA
UV	Ultraviolet
dNTPs	Deoxyribonucleotide triphosphate
w/v	Weight per volume
OD	Optical density
bp	Base pair

ABSTRAK TESIS

AMPLIFIKASI GEN 'ZINC FINGER PROTEIN' SEBAGAI TANDA PENGENALPASTIAN SPESIS LEMBU MALAYSIA

Teknik PCR-RFLP dibangunkan untuk mengenalpasti tiga spesis lembu tempatan, Lembu KK, Lembu Bali dan Seladang. DNA genomik daripada enam sampel lembu dipencilkan daripada persediaan leukosit. Amplifikasi PCR terhadap DNA genomic tersebut menggunakan primer-primer spesifik gen zfx; P1-5EZ dan P2-3EZ telah menghasilkan fragmen-fragmen bersaiz kira-kira 420bp daripada semua sampel. Selepas analisis pemotongan, enam enzim ditentukan untuk digunakan dalam membezakan ketiga-tiga spesis lembu berkenaan. Enzim tersebut adalah *Bpm* I, *Nla* IV, *Hin* 4I, *Taq* II, *Tsp RI* adan *Alu* I. Ketiga-tiga spesis lembu dapat dibezakan dengan menggunakan enzim spesifik yang hanya memotong satu urutan DNA zfx untuk speies tersebut. *Bpm* I dan *Nla* IV boleh momotong 416 bp fragmen lembu KK tetapi tidak memotong pada tapak urutan DNA dalam dua spesis yang lain. Kedua-dua enzim ini menghasilkan satu tapak pemotongan yang unik dan menjadi tanda pengenalpastian untuk lembu KK. *Hin* 4I dan *Taq* II boleh memotong 419 bp urutan DNA zfx lembu Bali dan secara langsung membezakannya dengan yang lain. Tiada enzim yang spesik dapat membezakan Seladang dengan spesis yang lain. Jika tiada pemotongan oleh empat enzim yang dinyatakan, kemungkinan sampel itu adalah dari Seladang. *Alu* I dan *Tsp RI* boleh digunakan untuk tujuan kepastian. Kedua-dua enzim memotong semua aturan DNA zfx daripada ketiga-tiga spesis lembu berkenaan tetapi pada tapak posisi yang berlainan. Pemotongan menggunakan kesemua enzim tersebut menghasilkan satu tanda spesifik untuk mengenalpasti spesis lembu. Teknik ini boleh

digunakan secara meluas dalam ujian saringan siasatan ke atas industri makanan, membantu dalam plan pemulihan pembiakan lembu dan mengenalpasti anak lenbu yang baru lahir.

ABSTRACT OF THESIS

AMPLIFICATION OF ZINC FINGER PROTEIN GENE (ZFX)
AS IDENTIFICATION MARKER FOR MALAYSIA BOVINE SPECIES

PCR-RFLP technique was developed to identify three endogenous bovine species; KK Cattle, Bali Cattle and Seladang. Total genomic DNA from six bovine samples was isolated from leukocyte preparation. PCR amplification of these genomic DNA using P1-5EZ and P2-3EZ primers for zfx gene resulted in a fragment approximately 420bp in size from all samples. After restriction analysis, six enzymes were determined to be used to distinguish all the three species. The enzymes include *Bpm* I, *Nla* IV, *Hin* 4I, *Taq* II, *Tsp RI* and *Alu* I. Three bovine species can be distinguished from each other by using specific enzyme that cut only one zfx sequence of the species. *Bpm* I and *Nla* IV could cleave the 416bp fragment in KK cattle, but there was no cutting site in the other two species. These two enzymes produce a unique restriction site and used as identification marker for the KK cattle. *Hin* 4I and *Taq* II could cleave the 419bp zfx sequence of Bali cattle and directly distinguish it among others. There was no specific enzyme for Seladang. If there was no cut by the four stated enzyme, the probability of the sample was Seladang. *Alu* I and *Tsp RI* enzyme could be used for confirmation purpose. Both enzymes cleaved all zfx sequence of the three bovine species but at different position. Digestion using all the enzymes produces a specific marker for identification of bovine species. This technique can be widely used for screening test in investigation of industrial food, support conservation cattle breeding plans and identification of newborn cattle.

1 INTRODUCTION

1.1 *Bos javanicus* (Bali Cattle)

Three subspecies of Banteng has been identified by the IUCN/SSC Asian Wild Cattle Specialist Group (Byers *et al.*, 1995). The list of current subspecies is Burma Banteng (*Bos javanicus birmanicus*), Javan Banteng (*Bos javanicus javanicus*) and Kalimantan (Borneo) Banteng (*Bos javanicus lowii*)

International Union for Conservation of Nature and Natural Resources (IUCN) Red Data List and the U.S Endangered Species Act classify the Banteng into endangered species. Population of the species decline of at least 20% over the last three generations with estimated current population is 2.3 million. People realize that the extinction of this species is because intensive crossbreeding programs using natural mating and artificial insemination. Conservation program has been intensively held in order to protect them from extinction.

Taxonomy

Kingdom	: Animalia
Phylum	: Chordata
Class	: Mammalia
Order	: Artiodactyla
Family	: Bovidae
Genus	: *Bos*
Species	: *Bos javanicus*

Figure 1.1: *Bos javanicus*

1.2 *Bos gaurus* (Seladang)

Gaur, also known as Indian bison or seladang is wildlife cattle. It is one of the biggest wild animals that populate in South and Southeast Asia. It has different kind of subspecies which populate in some country. According to the Asian Wild Cattle Conservation Assessment and Management Plan, three wild subspecies are generally recognized, including *Bos gaurus laosiensis* (Myanmar to China), *Bos gaurus hubbacki* (Thailand and Malaysia) and *Bos gaurus gaurus* (India and Nepal).

Global population of wild gaur ranges from 13,000 to 30,000 only (National Research Council, 1983). In Peninsular Malaysia, it is estimated that there are only 300 to 500 individuals left here. This animal is currently classified among endangered species and listed as vulnerable by IUCN. Our government has set up a place where the animal is intended to be conserved at Jenderak Seladang Sanctuary, Pahang.

Taxonomy

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Figure 1.2: *Bos gaurus*

1.3 *Bos taurus* (Kedah-Kelantan cattle)

Kedah-Kelantan cattle or other name is KK cattle is actually coming from species of normal cattle, *bos taurus.* There is disagreement about the hereditary of this species because there was no research that proofs it yet. However, it is more related to Thailand Native cattle and Yellow Cattle from China. This KK cattle is widely distributed in this country and being one of the source of meat product for industrial food.

Figure 1.3: *Bos taurus*

IUCN has classified both Seladang and Banteng as endangered bovine species. The violent killing of cattle for meat, skin hair, horn products and are critical in some country. During 1991–1995, 120 wild gaurs from different generation were reported to be killed in Vietnam In Thailand such as Meru Betiri NP and Alas Purwo NP, poaching and habitat destruction are still serious threat to Banteng population. Small populations of banteng in northern Australian are heavily relied on as a source of income for sport hunting as well as aboriginal peoples (IUCN Red List of Threatened Species).

It is necessary to have a molecular biology assessment by developing a marker among local cattle species for identification purpose. This maker is very helpful in

detection of the cattle species in various type of sample like skin, tissue, milk or meat during investigation process. This technique can be an enforcement tool in food industry to avoid from discrimination of cattle species especially gaur which has been manipulated by human business activities. The genetic marker were developed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Zinc finger protein gene (zfx) located on X chromosomes is amplified using PCR and then the product is digested by restriction enzymes to produce different length of fragments which can be observed in agarose gel electrophoresis. This polymorphism of fragment length uses an identification marker for the cattle's species.

2 LITERATURE REVIEW

2.1 Characteristic of Zinc Finger Protein gene (ZFX)

Zinc finger protein is a group of protein that binds to double helix DNA which consists of finger protein and zinc ion on the structure. It has diversity of function but it mostly found to role in development regulation either transcriptional activator or repressor depending on physiological context (Wingender, 1993). The integrity of zfx has been shown to be crucial for growth during embryogenesis and sustained gamete production (Gazin, 1999). Many of genes encode for this proteins are clustered in certain regions of genome (Wingender, 1993). In sex chromosome, the zinc finger protein gene on Y chromosome (zfx/zfy) encodes 13 finger proteins structure that located in 'sex determining region' of the chromosome (Page *et al.*, 1987). For X chromosome, the gene is homologous gene (zfx/zfx) in human (Schneider-Gadicke *et al.*, 1989).

Most of gene content in X chromosome is almost completely conserved between species. The study was done with comparison between human and mouse (Graves *et al.*, 1995). The zfx gene is one of the X-linked genes (Adler *et al.*, 1991). It is ubiquitously transcribed and highly conserved among vertebrates (Gazin, 1999). This feature can thus be used to amplify the gene for bovine species and consequently useful for generating a genetic marker.

The zinc finger protein gene was reported to be 79% homologous to similar gene (*Zfy*-1) on mouse (Ashworth *et al.*, 1989). Oligonucleotide primers were designed based on the gene sequence of mouse *Zfy*-1 gene (Aasen and Medrano, 1990). It is possible to amplify about 440 bp (zfx) fragments from bovine species by choosing primers from

sequences that are conserved between human ZFY, ZFX genes and mouse *Zfy*-1, *Zfy*-2 genes. By using these sets of primer, part of zfx gene is amplified but maybe in different size.

P1-5EZ	5-ATA ATC ACA TGG AGA GGC ACA AGC T-3
ZFX	.....C.....C.....
ZFY	...C.....C.....C.....
Zfy-1	.....
Zfy-2	.....
P2-3EZ	5-GCA CTT CTT TGG TAT CTG AGA AAG T-3
ZFX	.....
ZFY	.....G.....G.....
Zfy-1	...TC....G.....
Zfy-2	...TC....G.....

Table 2.1: Oligonucleotide primers P1-5EZ and P2-3EZ compared to the sequences of human ZFX and ZFY and to mouse *Zfy*-1 and *Zfy*-2 (Aasen and Medrano, 1990)

2.2 PCR-RFLP as identification marker

Polymerase chain reaction (PCR) technique and direct sequence analysis is a very useful and effective for species identification study. Most popular technique widely used that apply in biology, medicine and food science is PCR restriction fragment length polymorphism (PCR-RFLP) technique (Kurihara *et al.*, 1999; Russell *et al.*, 2000; Sato *et al.*, 1998). Previous study showed the PCR-RFLP has been utilized for rapid detection of meat product using mitochondrial and chromosome genetic sequences as marker (Maede, 2006). In this study, zfx gene has been analyzed and developed as genetic marker to identify three endogenous bovine species in Malaysia for multipurpose especially to identify imported meat in industrial food. The amplification of zfx region was proven in human, sheep, goat and cattles, but not in horses and pigs (Aasen and Medrano, 1990).

PCR is actually a technique that amplifies DNA sequence of gene exponentially. It is a molecular technology that has been invented by Kary Mullis (K.B. Mullis, U.S. patent 4, 683, 195 July 1987). The application of this technique has evolved through out research community and being used for development of multiple purposes. But recently, PCR is used to clone a given DNA sequence *in vitro*, without using living cells during cloning process (Gardner *et al.*, 1989).

There are three steps that involved in PCR which the process is repeated many times to produce cycle amplification. First is denaturation of genomic DNA which it is applied by heat at certain temperature to produce DNA templates. Then, the denatured DNA is being annealed by the oligonucleotide primers and finally DNA polymerase is used for replication of the DNA segment between sites of complementary to the primers. The product of the first cycle the being new DNA template and being amplify through next

cycle. The process repeated many times until finish the desired cycle. At the end, the size of the amplified molecules can be determined by gel electrophoresis.

In this study, PCR was used to amplify the zfx gene on X chromosomes from three bovine species; KK cattle, Bali cattle and seladang. The PCR product was digested by specific restriction enzymes to produce different length of fragments which can be observed in agarose gel electrophoresis. This polymorphism of fragment length was used as identification marker for the cattle's species.

3 MATERIALS & METHODOLOGY

3.1 Blood collection

Six total samples were collected from the cattles which two samples for each species. The fresh blood was collected selectively from different conservation place based on location of the cattle. Two livestock seladang at Jenderak Seladang Sanctuary, Pahang were chosen to be research sample with female selection only. About 7mL of blood was collected from female Seladang (Seroja and Awani) using sterile syringe and transferred into heparin-anticoagulated tube to ensure that no blood clots occur. Same procedure was practiced for Bali cattle and KK cattle samples before all the samples were stored at -20°C in freezer. Sample for Bali cattle was taken from Segamat, Johor while sample for KK cattle was taken from Kampong Lundang, Kelantan. Collection procedure was done by veterinary officer for safety purpose.

3.2 Extraction and purification of genomic DNA (Maniatis *et al.*, 1989)

First, about 2.5mL of blood sample was added with one volume of PBS. The mixture was slowly vortexed before centrifuged at 4000rpm, 4°C for 30 minutes and the supernatant was discarded after finished. The pellet was suspended in one volume of extraction buffer (10mM Tris Cl pH 8.0, 0.1M EDTA pH 8.0 and 0.5% SDS).The mixture was incubated at 37°C for one hour. Incubation continued at 50°C for 3 hours or overnight after proteinase K (100μg/mL) was added into the mixture.

After digestion, the sample was extracted three times with phenol and twice with chloroform. The extract precipitated after mixed with 0.2 volumes 10M ammonium acetate

and 2 volumes 100% cold ethanol. DNA precipitate can be observed in the tube. In order to get the DNA, the mixture was centrifuged at 4000rpm, 4°C for 20 minutes. The dried pellets were resuspended in TE buffer and stored at -20°C.

Because conventional DNA extraction method was sensitive to extract DNA old blood, a recent developed kit (i-genomic blood extraction mini kit) was applied in this study. 200µL of old blood was extracted step by step using buffers that were provided. For cell lysis, 20µL of proteinase K, 1µL of RNase A and 200µL of buffer BG was treated on sample before incubated at 65°C for 10 minutes. After that, 200µL of buffer BB was added into mixture before spin at 13 000rpm for 10 minutes. The mixture was washed with 700µL of buffer BWA and BWB. The DNA was eluted in 50µL of elution buffer. Total DNA extracted was about 100µg from each sample