UNIVERSITI PUTRA MALAYSIA

GENETIC IDENTIFICATION OF PUREBRED AND CROSSBRED WATER BUFFALO [*Bubalus bubalis* (LINNAEUS, 1758)] IN SELECTED FARMS IN MALAYSIA

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By

NOR 'AMMAR LIYANA BINTI SHAARI

Thesis submitted to the school of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement of the Degree of Master of Science

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DEDICATION

This thesis is especially dedicated to the most lovely parents in the world, my parents Shaari bin Harun and Che ‘Ah binti Ahmad and also my family. Besides that, I would also express my gratitude to all my lecturers especially to all my committee supervisors. A lot of thanks to all my friends that give me full supports and encouragement. They include the nutrition lab family, biochemical lab family and postgraduate family.

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

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March 2019

Chairman : Hafandi bin Ahmad, PhD
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Water buffalo or *Bubalus bubalis* is a domesticated animal that plays essential roles in agriculture, economy and food production. Water buffalo is classified into two sub-species based on the phenotypic, genotypic and cytogenetic. The two subspecies of water buffalo is murrah buffalo *(Bubalus bubalis bubalis)* and swamp buffalo *(Bubalus bubalis carabensis).* Identification based on morphology is not enough to confirm the species or to make the comparison between the two sub-species. The problem arises when these two sub-species mates and produce their crossbreed offspring, which is indistinguishable from their parents. Therefore, the aim of this study is to determine three sub-species of water buffalo available in Pusat Ternakan dan Pembiakan Kerbau, Telupid, Sabah and Ladang Ternakan Kerbau Semenyih (Semenyih Farm) using karyotyping and molecular methods. In addition, phylogenetic analysis is to determine the differences in the nucleotide sequences, associated with the relationship evolutionary events between species. The blood of 112 animals were taken, cultured, terminated and harvested using conventional karyotyping protocol to obtain the chromosomes. Results showed that from 112 buffaloes in PLadang Ternakan dan PembiakanHaiwan, Telupid, Sabah, 97 animals successfully identified were confirmed as swamp buffalo presented with 48 chromosomes and the remaining 7 were identified as crossbred buffalo presented with 49 chromosomes. However, in Semenyih Farm, 8 animals were successfully identified as murrah buffalo presented with 50 chromosomes. In molecular work, the bloods were collected and the PCR were conducted to quantify the d-loop of mitochondrial DNA in three sub-species. Two pairs of primers known as NP1 and NP2 were designed to amplify the full d-loop region by PCR. The purified PCR products from three sub-species were then sent for sequencing. Results from sequencing showed that the full d-loop of mitochondrial DNA is successfully amplified, consist of 1000 base pairs. Based on the multiple sequence analysis, the three subspecies of water buffalo shared many conserve region and they also showed some differences in their nucleotide sequences. The mean

molecular weight (Da) is also differ each sub-species, which swamp with 343,747.70 Da, crossbreed with 345,331.30 Da and murrah with 335,374.22 Da. All d-loop mitochondrial DNA sequences from this study hit the NCBI blast for the most similar sequences with 99% identical to the sequences available from the database. Phylogenetic tree was constructed using maximum likelihood analysis using software MEGA 7.0. From the phylogenetic results, the swamp and murrah clearly formed two different clades, showing that they are diverged from each other. On the other hand, crossbreed buffalo is claded into the swamp buffalo, showing that swamp buffalo is more dominant in the crossbreed compared to murrah buffalo. By combining karyotyping method, molecular study and also phylogenetic tree, the result obtained from this study is more convincing and complete compared to analysis done solely on one aspect. In conclusion, the genetic identification is crucial in identifying the three sub-species of water buffalo, as it can provide more concrete and clear result compared to conventional method such as morphological analysis.

Abstrak thesis ini dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENGENAL PASTIAN GENETIK KERBAU BAKA ASLI DAN KACUKAN [*Bubalus bubalis* (LINNAEUS, 1758)] DI LADANG-LADANG TERNAKAN TERPILIH DI MALAYSIA

Oleh

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Kerbau atau nama saintifiknya *Bubalus bubalis* adalah sejenis haiwan domestik yang memainkan peranan penting dalam sektor pertanian, ekonomi dan penghasilan makanan. Kerbau dibahagikan kepada dua sub-spesies berdasarkan finotipik, genotipik dan sitogenetik. Dua sub-spesies tersebut adalah kerbau sungai *(Bubalus bubalis bubalis)* dan kerbau sawah (*Bubalus bubalis carabensis).* Pengenalpastian berdasarkan morfologi sahaja tidak mencukupi untuk memastikan kesahihan sesuatu spesies, atau untuk membuat perbandingan di antara dua sub-spesies tersebut. Masalah mula timbul apabila dua sub-spesies kerbau mengawan dan menghasilkan keturunan hibrid, yang sukar untuk dibezakan daripada ibubapa mereka. Oleh itu, tujuan kajian ini adalah untuk menentukan tiga sub-spesies dari kerbau yang terdapat di Ladang Ternakan dan Pembiakan Kerbau, Telupid, Sabah dan Ladang Ternakan Kerbau, Semenyih (Ladang Semenyih) menggunakan kaedah karyotiping dan molekular. Di samping itu, analisis filogenetik yang dialakukan adalah untuk menentukan perbezaan dalam jujukan nukleotida, yang berkaitan dengan hubungan evolusi peristiwa antara spesies. Darah daripada 112 ekor haiwan diambil, dikultur, ditamatkan dan dituai menggunakan protokol karyotiping konvensional untuk mendapatkan bilangan kromosom. Hasil kajian menunjukkan bahawa dari 112 ekor kerbau, 97 ekor haiwan yang berjaya dikenal pasti disahkan sebagai kerbau sawah yang dibentangkan dengan 48 kromosom dan baki 7 dikenalpasti sebagai kerbau hibrid yang dipersembahkan dengan 49 kromosom. Walaubagaimanapun, di Ladang Ternakan Kerbau Semenyih, 8 ekor haiwan yang berjaya dikenal pasti disahkan sebagai kerbau murrah. Dalam kerja molekular, darah dikumpulkan dan PCR dijalankan untuk mengukur d-gelung DNA mitokondria dalam tiga subspesies. Dua pasang primer yang dikenali sebagai NP1 dan NP2 direka bentuk untuk menguatkan rantau gegelung penuh-d oleh PCR. Produk PCR yang dari ketiga-tiga sub-species telah dibersihkan dari impuriti itu kemudiannya dihantar untuk penjujukan. Gegelung DNA mitokondria penuh berjaya dikuatkan, terdiri daripada 1000 pasangan asas. Berdasarkan analisis jujukan

berganda, tiga sub-spesies kerbau berkongsi banyak kawasan yang sama dan mereka juga menunjukkan beberapa perbezaan dalam jujukan nukleotida antara mereka. Purata berat molekular(Da) adalah berbeza-beza bagi setiap sub-spesis, di mana kerbau sawah ialah 343,747.70 Da, baka campuran ialah 345,331.30 dan murrah dengan 335,374.22 Da. Kesemua jujukan dari gegelung d di dalam mitokondrial DNA daripada kajian ini menyamai Blast NCBI bagi kenbanyakan spesis lain dengan nilai sebanyak 99% kesamaan kepada jujukan yang boleh didapati di dalam databes. Pokok Phylogenetic dibina menggunakan analisis kemungkinan maksimum menggunakan perisian MEGA 7.0. Dari hasil filogenetik, kerbau paya dan murah jelas membentuk dua klad yang berbeza, menunjukkan bahawa mereka tersebar dari satu sama lain. Sebaliknya, kerbau hybrid dipasangkan ke dalam kerbau paya, menunjukkan bahawa kerbau paya lebih dominan berbanding kerbau murah. Dengan menggabungkan kaedah karyotiping, kajian molekular dan juga filogenetik, hasil yang diperoleh daripada kajian ini lebih meyakinkan dan lengkap berbanding dengan analisis yang dilakukan hanya pada satu aspek. Kesimpulannya, pengenalan genetik adalah penting dalam mengenal pasti tiga sub-spesies kerbau air, kerana ia dapat memberikan hasil yang lebih konkrit dan jelas berbanding dengan kaedah konvensional seperti analisis morfologi.

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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 Supervisor Committee were as follows:

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LIST OF ABBREVIATIONS

A	Adenine
AFLP	Amplified fragment length polymorphism
BLAST	Basic Local Alignment Software Tool
Bp	Base pair
C	Cytokinine
Cm	Centimetre
CTAB	Cetyl-trimethylammonium bromide
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DNTPs	Deoxyribonucleoside triphosphate
EDTA	Ethylenediamnetetraacetic acid
EtOH	Ethanol
G	Guanine
HCL	Hydrochloric acid
KCl	Potassium chloride
Lb	Pound
$MgCl_2$	Magnesium chloride
Ml	Milimitre
MW	Molecular weight
NaOAC	Sodium Acetate
NaOH	Sodium Hydroxide
NCBI	National Centre Biotechnology Institute
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
RAPD	Random Amplification of Polymorphic DNA
RNA	Ribonucleic acid
SNP	Single-nucleotide polymorphism
T	Thiamine
TAE	Tris-acetate
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Water buffalo is a domesticated animal that is postulated to be domesticated in the Indus civilization about 5000 years ago (Sangwan, 2012). Nevertheless, water buffalo was domesticated in China at the time of the fifth millennium before century which was occurred earlier compared to India (Chen and Li, 1981). However, according to Mason (1974), water buffalo was extensively scattered during the Pleistocene era, although at a later time the species was only limited to the India subcontinent and Southeast Asia. Generally, there are two types of water buffaloes which have been categorized based on morphological, behavioural, geographical and number of chromosomes (Sangwan, 2012).

The first type is African water buffalo which is scientifically named *Syncerus caffer* and the second type of water buffalo is commonly called Asiatic water buffalo which is scientifically named *Bubalus bubalis.* The *Syncerus caffer* buffalo is further categorized into two genus which are *Syncerus caffer caffer* and *Syncerus caffer nanus* (Van Hooft *et al*., 2002). African water buffalo is not domesticated animal due to its erratic behaviour which makes it harmful for human being. Nonetheless, *Bubalus bubalis* or Asiatic water buffalo is also categorized into two main groups which are *Bubalus bubalis bubalis* and *Bubalus bubalis carabensis*. On the contrary, *Bubalus bubalis* is a significant tool which not only serves as livestock animal but gives benefit in agriculture, economy, food production and also vital in some cultures (Harisah., 1988). In agriculture sector, *Bubalus bubalis* is being used as a draft animal or transport that assist human in the field or farm (Yindee *et al*., 2010).

In Malaysia, two subspecies of water buffalo (*Bubalus bubalis*) are commonly known as murrah and swamp buffaloes. Morphologically, swamp buffalo is smaller in size and usually has slate blue or grey in colour (Borghese, 2005). However, murrah buffalo is bigger in size with pronounced horn and black jet in colour. On the other hand, swamp buffalo loves to wallow in murky water such as swamp thus contributing to their name (Borghese, 2005; Thiruvenkadana, 2013). Murrah or "river" buffalo usually preferred wallow in clean water such as river water thus giving them the name. Swamp buffalo is usually reared mainly for draught purposes especially in paddy field and oil palm plantation, although it also produced a valuable milk yield of up to 600 kg milk per year (Cruz, 2010; de la Cruz-Cruz, 2014). On the other hand, murrah buffalo is reared specifically for their milk and meat which is due to the massive production of milk and meat compared to swamp buffalo (Borghese, 2005).

Identification based on morphology and identification from local name is not sufficient to confirm the species, or to make the comparison between the two subspecies. The problem rise when these two subspecies are mating and produced their crossbreed offspring, which is difficult to identify from their parents. Thus, the identification of animals based on the karyotyping is the first step for identification, because we can compare the numbers and shapes of chromosomes directly in each individual animal. In addition, identification by molecular approach was widely used among scientist or researches, because it provides them with detail confirmation. For example, mitochondrial DNA (mtDNA) was widely used as targeted region as there is usually no change in mtDNA from parent to offspring. The mutation rate of animal mtDNA is higher than that of nuclear DNA, thus making mtDNA a powerful tool for tracking ancestry through females (matrilineage) and has been used to track the ancestry of many species back hundreds of generations (Burgstaller *et al.*, 2014).

Moreover, the fast mutation rate makes mtDNA advantageous for assessing genetic relationships of animals or groups within a species (Burgstaller *et al.,* 2014). It is also useful for identifying and quantifying the phylogeny among different species. In fact, mtDNA avail to measure the relationship between both closely related and distantly related species. Due to the high mutation rate of mtDNA in animals, therefore providing the information about the genetic distances among closely related individuals or species (Burgstaller *et al.,* 2014). On the other hand, because of the substitution rate of mt-proteins is very low, thus amino acid changes accumulate will give the information about the genetic distances of distantly related species (Taylor *et al*., 2005).

1.2 Problem statement

This study aimed to overcome the limited sources of water buffalo in the termof genetic especially in Malaysia. Mostly, water buffalo was being studied mainly on the behaviour and morphology only while the genetic underlying of water buffalo is still lacking. In conclusion, this study will provide researches with better understanding on the genetic underlying of water buffalo in Malaysia.

1.3 Significant of the study

Water buffalo is distributed widely around the world starting from India, China, Philippines, Malaysia and also across continents in Italy and Australia (Borghese, 2005; Kandeepan *et al.,* 2009). In Malaysia, water buffalo has great potential for meat and milk production, especially in the state of Sabah and a few states in Peninsular Malaysia such as Selangor, Perak and Pahang (Ariff *et al.*, 2015; Department of Veterinary Services Malaysia, Livestock Statistic, 2016). This could indicate that water buffalo is less commonly consumed by the community compared to cattle, sheep or goat. There are several factors that contribute to the decrease in demand such as low multiplication rate, long generation interval and high extraction rate. In fact, the lack of effort and priority given by the government to the buffalo industry has also led to the decline in demand and population.

It has been established that identification of water buffalo species are by morphology and morphometric methods is common and not accurate in terms of the genetic application. In addition, study done using the molecular approach will implement all the underlying genetic information of water buffalo that will then be used to construct the phylogenetic tree. Furthermore, information from phylogenetic tree will then give researchers the estimation in the relationship of the three subspecies of water buffaloes available in Malaysia. However, lack of information from previous research about the bioinformatics population and genetic underlying of water buffaloes can be overcome by doing this study. Therefore, through this identification, we should be able to know the background of water buffaloes and classify them based on their breeds.

1.4 Objectives of the study

Therefore, the objectives of this study are;

1) To identify the purebred of water buffaloes and their crossbreed available in Ladang Ternakan Kerbau, Telupid, Sabah and Semenyih Farm through karyotype technique.
2) To isolate the d-loop of mitochondrial DNA (mtDNA) of purebred water buffaloes and their crossbreed available in Malaysia.
3) To determine and estimate the relationship between the purebred water buffaloes and their crossbreed using phylogenetic analysis

1.5 Hypotheses of the study

It was hypothesized that;

Swamp buffalo is presented with 48 chromosomes, crossbreed buffalo is presented with 49 chromosomes and murrah buffalo was presented with 50 chromosomes, while alternative hypothesis of swamp buffalo, murrah buffalo and crossbreed buffalo will not be presented with 48 chromosomes, 50 chromosomes and 49 chromosomes respectively.

D-loop mitochondrial DNA of water buffalo sequences in all three subspecies of water buffalo are estimated to be around 1000 base pairs, while alternative hypothesis might be that d-loop mitochondrial DNA of water buffalo sequences in all three subspecies of water buffalo might not produce result around 1000 base pairs.

Phylogenetic tree of water buffalo will form two different high variability clades containing murrah, swamp and crossbreed buffaloes, while alternative hypothesis might produce single clade of water buffalo only.

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Throughout her master study, she participated in a lot of seminar, workshops and conferences related to the fields of study. Furthermore, she had presented her poster presentation at the 12th Malaysia International Genetics Congress 2017 (MiGC12) which will be held in Hotel Bangi-Putrajaya, Malaysia from 25-27 September 2017. In addition to that, she also presented her papers at the International Seminar on Livestock Production and Veterinary Technology on October 16-17th, 2018 in Kualanamu, Medan.

LIST OF PUBLICATIONS

Proceedings

Liyana-shaari, N.A., Zamri-saad, M., Rosnina, Y., Izza, A.G.N., Shahrom, S., and Hafandi A. (2017). Identifications of Water buffalo in Ladang Ternakan Haiwan, Telupid, Sabah by karyotyping. Oral presentation at 12th Malaysia International Genetics Congress 2017 (MiGC12) which will be held in Hotel Bangi-Putrajaya, Malaysia from 25-27 September 2017.

Liyana-shaari, N.A., Zamri-saad, M., Rosnina, Y., Izza, A.G.N., Shahrom, S., Marilyn J., Loo S.S., and Hafandi A. (2018). Identification of Water Buffalo through Cytogenetic and d-loop Mitochondrial DNA. International Seminar on Livestock Production and Veterinary Technology on October 16-17th, 2018 in Kualanamu, Medan.

Research articles

Liyana-shaari, N.A., Zamri-saad, M., Rosnina, Y., Izza, A.G.N., Shahrom, S., Marilyn J., Loo S.S., and Hafandi A. (2018). Genetic Characterization of Water Buffalo in Malaysia. *BMC Genetic*.

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