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PHYLOGENETIC ANALYSIS OF LOCAL ENDEMIC BUFFALO (*BUBALUS BUBALIS*) BASED ON CYTOCHROME B GENE IN CENTRAL INDONESIA

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Graphical abstract

Abstract

Buffalo population in the central of Indonesia such as Bali, NTB, and Sulawesi is decreased. Cause of the decline of buffalo population is inbreeding. For conservation efforts to avoid inbreeding required the identification of buffalo genetic variations on molecular scale as phylogeny analysis with cyt b gene. The purpose of this study were to reconstruct the phylogenetic tree of buffalo ((Bubalus bubalis) in the central of Indonesia with cyt b gene. Animal materials were collected from four different area: Bali, Lombok, Bima and Toraja. DNA isolation, PCR (polymerase chain reaction) was conducted under standar protocols. Sequence from four representaive animals based on area were used to contruct the phylogenetic tree and haplotype network. Analyses of plylogenetic tree showed that the reconstruction has one major clade and two sub clade. It showed that buffalo in central of Indonesia had close genetic relationship.

Keywords: Bubalus bubalis, cytochrome B gene, phylogenetic tree

1.0 INTRODUCTION

Local buffalo (*Bubalus bubalis*) is a ruminant that has high benefit for Indonesian people. Among these benefits are to plow the field and as a source of animal protein. Despite having great benefits, a fact proved that in recent years in some areas of the central part of Indonesia such as Bali, West Nusa Tenggara and Sulawesi, the buffalo population has decreased. Based on the average percentage of growth buffalo population has decreased by 0.58% per year from 2003 to 2011 [1]. It was redicted that cause of the population declining is inbreeding [2]. The negative impact of inbreeding is to reduce individual profitality [3]. Therefore, it requires genetic variations identification of molecular buffalo in order to avoid the occurrence of inbreeding as conservation efforts with regard relationship.

Recently studies informed the identification of genetic variations buffalo based on microsatellites as molecular markers have been conducted at the Laboratory of Molecular Biology, State University of Malang. The data included heterozygosity, PIC (polymorphism information contents) value and allele frequencies in buffalo population in central Indonesia.

2.0 LITERATURE REVIEW

The mitochondrial cytochrome-*b* gene has been a popular source of DNA sequences for phylogenetics reconstruction though few studies have considered the evolutionary dynamics of this gene and encoded protein [4]. There are great functions of cytochrome-*b,* such as to analyze structure and evolution of opposum, guinea pig and porcupine [5]; pathway of lysozyme evolution in birds [6]; accelerated evolution in simian primates [7]; phylogenetic analyses of order carnivora [8]; genetic relationships

of Japanese and Korean Bagrid Catfishes [9]; phylogenetic relationship among east asian species of Crocidura/Mammalia-Insectivora [10]; phylogenetic relationship among Asian species of Petaurista/Rodentia and flying squirrel genera [11].

In the early 1990, the first set of versatile primers was published for portion of the cytochrome-*b* gene, the small ribosomal RNA gene, and the major non-coding region. These primers were found to amplify homologous portions of mtDNA in all classes of vertebrates and many other animal phyla (Meyer 1994). In the phylogenetic research, it was usually utilized a sequence comparison, because sequence comparison is a powerful tool for understanding processes and pattern of nucleotide substitution and how these might impinge on phylogenetic inference.

The relationship of buffalo population in central Indonesia could be represent by phylogenetic tree. The partial cytochome b gene was choiced as a molecular markers. Some reason of using is the maternal inheritance models [12]. The gene cytochome b is the best sample of mitochondrial DNA to mammals. Fragments of genes cytochome b quite able to distinguish closely related species [13].

The aim of the study was to describe the genetic variation in DNA buffalo (*Bubalus bubalis*) from the central part of Indonesia based on cytochome b gene to reconstruct the phylogeny tree, and also similarity sequence, genetic distance, and variant.

3.0 METHODHOLOGY

3.1 DNA Samples

Blood samples from eight local Buffalo were collected and preserved in EDTA solution/powder (obtained from five different areas). Blood samples were taken from farmers. DNA was isolated from the whole blood using standard SDS/proteinase K methods and phenol/chloroform extractions to get a genome DNA [14]. Isolation of genomic DNA by GenomeClean ™ method includes the following steps. Lysis: Mix 0.6 ml Buffer 1 with 0.3 ml sample (whole blood, up to 150 mg tissue) in a 2 ml reaction tube. Lysis is performed by incubation at 68 ◦ C for 5-10 min. Deproteinization: Add 0.9 ml Chloroform and gently mix until the two phases have formed an emulsion. Separate the two phases by centrifugation at 10.000 rpm for 2 min at room temperature in a microfuge. DNA precipitation: transfer the upper aqueous phase containing the genomic DNA to a new 2 ml reaction tube and precipitate DNA by adding 0.9 ml Aquabidest and 0.1 ml Buffer 1. After gently mixing by inversion at room temperature isolate precipitate DNA using a glass rod or centrifuge at 10.000 rpm for 2 min at room temperature. DNA resuspension: for exchanging the detergent resuspend precipitate DNA in 0.3 ml Buffer 3. DNA precipitation: add 0.75 ml 96 Ethanol and gently mix by inversion. Centrifuge sample at 10.000 rpm for 10 min at room temperature. Discard supernatant and rinse pellet with 70% ethanol. After the removal of 70% ethanol, dissolve the DNA in TE-buffer.

3.2 PCR

PCR was carried out by all sources under standard condition. A 307 bp fragment of the cytochrome b genes was amplified using "universal" primers:
Cytb L 14841:
5'AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA 3'
Cytb H 15149:
5'AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA 3'
Annealing temperature: 50-54°C
Parameter: Denature at 90 °C 60 seconds
Anneal at 52 °C 60 seconds
Extend (elongation) at 72 °C 90 seconds.
Repeat over 30 – 35 cycles.

3.3 Sequencing

Sequence of DNA obtaine from PCR product and conducted by Genetic Science Jakarta. Nucleotide sequence of partial cytochrome-*b* were prepared and aligned manually. The numbers of variable sites and phylogenetically informative sites were determined using MEGA [15]. Phylogenetic relationships to tree reconstruction were assessed using cytochrome-*b* nucleotide sequences. Sites of sequence alignment showing insertion/deletions (indels), missing data, and ambiguities were excluded from phylogenetic analysis. Neighbor-Joining (NJ) analyse was performed using MEGA [15].

Biogeography analysis in form of haplotype network was reconstruct by using network 4.6.1.3 software.

4.0 RESULTS AND DISCUSSON

In this study, the *cytohrome b* genes are successfully amplified by *polymerase chain reaction* (PCR) approximately 307 bp. PCR product was shown in Figure 1.

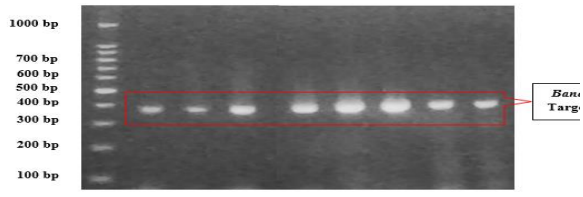

Figure 1 The results of PCR from cytochrome b gene. The band obtained ± 350 bp (base pair). M = Marker, F2-F20 = Toraja, 23AB-22A = Bali, AB7-WE4 = Bima, LS2-LM6 = Lombok

Data from eight sequences compared to sequences *Bison bison* as out group were analyzed

using the online program MEGA 6.0 software. It was used to reconstruct of phylogeny tree. Phylogeny tree reconstruction was made based on the method Neighbour Joining (NJ) method and Minimum Evolution (ME) as a comparison. Both methods can create an evolutionary tree based on the evolution rate [16]. The phylogeny tree in NJ method produces one type of tree, while in the ME method, all of possible trees searched and compared to get good phylogeny tree [17]. Results of phylogenetic tree construction based methods NJ and ME were shown in Figure 2 and Figure 3.

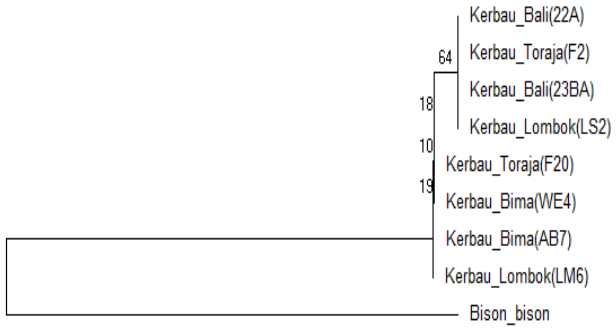

Figure 2 Phylogenetic tree of *Bubalus bubalis* in Central Indonesia and *Bison bison* as outgroup using Neighbor Joining (NJ) with a model of Kimura 2 parameter model and phylogeny Test Bootstrap 1000

Figure 2 and 3 showed the similar phylogenetic tree reconstruction with Neighbour Joining methods and Minimum Evolution (ME), and there is one major cluster and was divided into two large groups. Cluster A is a local buffalo in central Indonesia. Cluster B outgroup (*Bison bison* species). Cluster A consist of two groups. Group 1 consisted of buffalo Bali (22A), buffalo Toraja (F2), buffalo Bali (23BA), buffalo Lombok (LS2). The relationship level of figbuffalo Bali (22A) and buffalo Bali (23BA) is very close, because biogeography are in same area. Also in group 2, buffalo Bima (WE4) and buffalo Bima (AB7) has a close relationship. This is relevan to Sukri (2014) that the buffalo that was one area allows breeding between the populations of buffalo. In the first group, there are buffalo Toraja (F2) and buffalo Lombok (LS2). In the second group there are also Buffalo Toraja (F20) and buffalo Lombok (LM6). Geographically, Lombok and Toraja are separate region. The existence of the close relatedness between Lombok and Toraja buffalo caused by the activities of the distribution of buffalo Lombok to Toraja. Animal husbandry department Toraja take a supply of buffalo from other districts especially from provinces outside the island of Sulawesi , for example Nusatenggara Barat (Sumbawa region, Bima, and Lombok) [18].

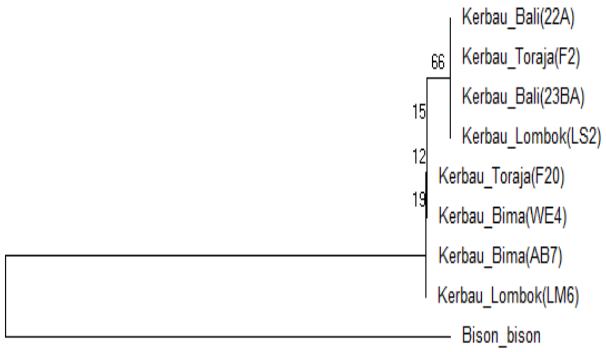

Figure 3 Phylogenetic tree of *Bubalus bubalis* in Central Indonesia and *Bison bison* as outgroup using Minimum Evolution (ME) with a model of Kimura 2 parameter model and phylogeny Test Bootstrap 1000

As a complement to further strengthen the data of reconstructed phylogenetic tree, analysis biogeography form of haplotype network to determine each sample were analyzed to cluster in one haplotype based biogeographic area. Construction of the haplotype network each sample of buffalo (*Bubalus bubalis*) in central Indonesian and *Bison bison* as a outgroup species using software Network 4.6.1.3. Biogeography analysis results was shown in Figure 4.

Figure 4 shows the number of haplotypes that formed was 9 haplotypes. Hap_1: buffalo Bima (AB7), Hap_2: buffalo Bima (WE4), Hap_3: buffalo Lombok (LM6), Hap_4: Lombok buffalo (LS2), Hap_5: Bali buffalo (23BA), Hap_6: Bali buffalo (22A), Hap_7: Toraja buffalo (F2), Hap_8: Toraja buffalo (F20), and Hap_9: Bison bison. Base on variations or positions of mutations that are formed between H_9 with H_1 until H_8 are so many but it also could be seen between H_9 with H_1 until H_8 formed many branch points. This shows H_9 close related to H_1 until H_8. H_9 is a outgroup species *Bison bison* that is endemic from America. The distance levels between buffalo is influenced by the location of the biogeographic are so far and it complicate the migration [14].

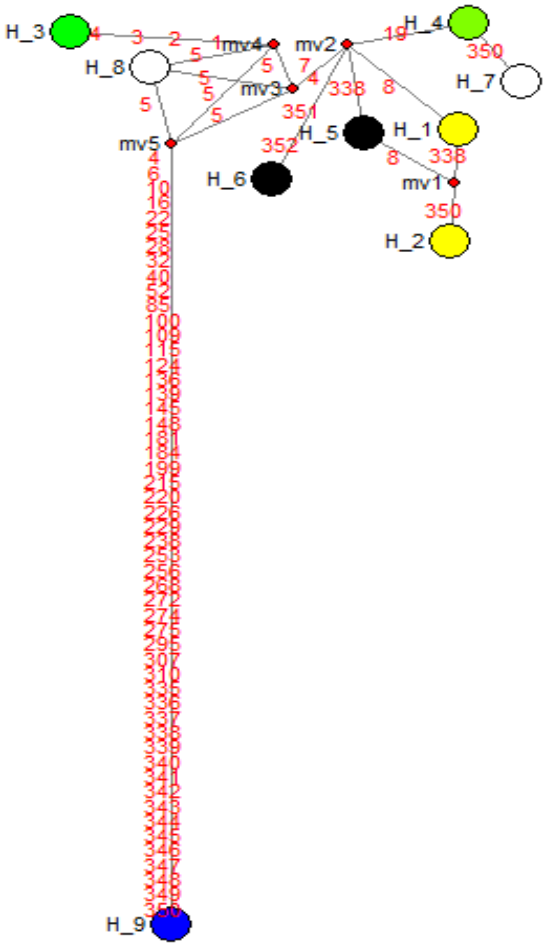

Figure 4 Haplotype analysis of the Buffalo Local in Central Indonesia and and *Bison bison*

5.0 CONCLUSION

Phylogenetic tree reconstruction methods NJ and ME for local buffalo in central Indonesia has close related and there was found 9 haplotypes

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