Phylogenetic Study of Madura Cattle Based on Mitochondrial Cyt b and D-loop Sequences

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**ABSTRACT**

Madura Cattle is one breed of local cattle from Indonesia. Madura cattle are estimated to originate from a crossbreeding between *Bos indicus* and *Bos javanicus*. Another presumption is that Madura cattle are the result of a crossbreeding between *B. indicus* males and mixed *B. javanicus* or *B. taurus*. Tracing the history of Madura cross and another cattle phylogenetic based on maternal lineage can be done by analyzing the variation of the mitochondrial genome (mtDNA). The purpose of this study was to determine the clarity of the origin of Madura cattle based on maternal lineage using mtDNA markers Cyt b and D-loop. This research is expected to provide genetic information and the origin of Madura cattle, so that it can be used to help improve the breeding and conservation program for Madura cattle. The results of the phylogeny tree reconstruction, using the Cyt b and D-loop genes showed that Madura cattle originated from Sampang region (Polagan, Golbung, and Komis) were grouped into two types of maternal origin. Madura cattle clade I are grouped with *B. indicus* and *B. taurus*, while Madura cattle clade II are grouped with *B. javanicus*. A crossbreeding between *B. javanicus* and *B. indicus* is estimated to have been carried out since the entry of Hindu culture brought by the India peoples to Indonesia around 1800 years ago. The crossing between *B. javanicus* and *B. indicus* was then more intensively carried out at the time of the government's promoting the development of Ongol cattles (*B. indicus*) in the days of the Dutch East Indies. The length segment of Cyt b that can be amplified is 230 bp and the D-loop segment of varying length, 577 bp for the Madura 41 and 29 samples, and 624 bp for sample 32.

Keywords: Cyt B, D-loop, Madura cattle, mtDNA, Phylogeny

**Introduction**

Cattle are animals that are the result of the domestication of wild cattle that have an important role in human life. The process of domestication has been ongoing from 4,000 to 5,300 years ago from wild cattle *Bos prigimineus* (McHugh, 1997; Mannen *et al.*, 1998). *Bos prigimineus* is domesticated into two types of cattle, there are *Bos taurus* and *Bos indicus*. These two species of cattle then developed into modern cows through the crossbreeding method. The cattle are crossed and their genetics are repaired as livestock. The species of cattle that develop as livestock without going through the crossbreeding process is *Bos javanicus*. *Bos javanicus* or banteng is one of the native Indonesian cattle which is different from *B. taurus* and *B. indicus*.

*Bos javanicus* as an Indonesian native cattle domesticated into Balinese cattle. Bali cattle are then spread throughout Indonesia. In addition to Bali cattle, there are also several of local cattle that develop as livestock in Indonesia, including Aceh cattle, Pesisir cattle, and Madura cattle. Aceh cattle and coastal cattle are from the *B. indicus* lineage which has a distribution area in the western provinces of Aceh and Sumatra. While Madura cattle have a limited distribution only on the Madura Island and the eastern part of Java. The origin of Madurese domestication of cattle is unclear and there are still many differences (Williamson and Payne, 1965; Uggla, 2008; Kusdiantoro *et al.*, 2009).

Madura cattle have morphological characteristics that are similar to the morphology of Balinese cattle. Madura cattle skin is reddish-brown with white motifs on the buttocks and legs. In addition to having similarities in morphological characteristics of cows Madura also has physiological characteristics that are similar to Balinese cattle which are more resistant to hot weather conditions, limited food conditions, have good meat quality, and are more resistant to...
certain types of parasites (Payne and Hodges, 1997).

The initial process of crossbreeding to obtain a stable Madura cattle line so far has not been recorded properly and there are still differences in some data from the research results. According to Williamson and Payne (1965) Madura cattle are thought to originate from a cross between B. indicus and B. javanicus. There is also a claim that Madura cattle are the result of a cross between B. indicus males and mixed B. javanicusor B. Taurus females. This is estimated because of the similarity of colors with Madura cattle, brownish red (Maksum, 1993). Kusdiantoro et al. (2009) study based on the SRY gene found that several samples of Madura cattle were descended from B. taurus.

Namikawa (1981) suspected that there was a mixture in Madura cattle. This is based on the type of hemoglobin (Hb-βx) in the blood of Madura cattle. The appearance of hemoglobin beta x (Hb-βx) in Madura cattle blood is thought to originate from B. javanicus. Hemoglobin βx has never been reported to appear on B. indicus or B. taurus.

The study of the history of Madura crossing and phylogeny can be done by analyzing variations in the mitochondrial mt (DNA) genome. Every individual who has the same brood will have the same mtDNA type. This is because mtDNA is inherited through the maternal line. Other advantages of using mtDNA are haploid (single copy) and do not experience recombination (Tapio and Grigaliunaite, 2002).

Based on this background, a study was conducted to find out the clarity of the origin or history of Madurese cattle based on maternal lineage using Cyt b and D-loop mtDNA markers. The results of this study can be used to help improve Madura cattle breeding and conservation programs. Madura cattle conservation efforts are still needed to enrich the assets of national germplasm considering Madura cattle are native cattle.

Materials and Methods

Sample collection
Madura cattle blood samples used in the study were taken from several regions in Sampang regency, Polangan village (sample no 14), Golbung (sample no 26, sample no 29, and sample no 32), and Komis (sample no 38 and sample no 41) (Figure 1). Blood samples as a source of DNA for each livestock were taken as much as 5-10 ml per animal through the jugular vein in the neck area of the body of the cow and then collected vacutainer tubes that had been given EDTA solutions as anti-coagulation and anti-microbial solutions.

Total DNA isolation
The sample obtained was extracted manually following the method developed by Sambrook et al. (1989) with a slight modification. Blood samples in alcohol were taken 300µl and deposited by centrifuging 5000 rpm for 10 minutes. The deposition of blood cells washed with distilled water and then deposited once more. The blood cells are then suspended in the STE lysis buffer (1M NaCl, TRI HCl-1M, EDTA 10-2 M, pH 8.0) to a volume of 300 µl. blood cells were then lysed with 0.05 mg/ml Proteinase K and 1% Sodium Dodecyl Sulfate, the mixture was shaken gently while incubated at 55°C for 1 hour. DNA molecules are separated from other organic materials by the phenol method, which is to add phenol 1x volumes and CIAA (chloroform: Isoamyl alcohol = 24: 1) 1x volume and 1/10x 5M NaCl volume. After gently shaking for 1 hour, the phenol phase was separated from the water phase by centrifuging 5,000 rpm for 10 minutes. The water phase in the upper layer of the phenol phase is transferred to the new tube with a measured volume. The DNA molecule is then deposited by the alcohol deposition method, namely by adding absolute 1M/10M NaCl volume and alcohol as much as 2x the volume phase of water. DNA deposits obtained after centrifuging 6,000 rpm for 10 minutes were then washed with 70% alcohol and then deposited again. The remaining alcohol is evaporated in the vacuum. The DNA deposits obtained are then suspended in the TE buffer (Tris-HCl 10 -1M EDTA 10-2M pH 8.0) 60µl and stored in the freezer until further work.

MTDNA amplification
Amplification of the mitochondrial genome using primary pairs AF22 (forward) 5’ GCGTACGCAATTCTAGATCA- 3’ and AF23 (reverse) 5’ ATGAGTTAAGTCCAGCTAC-3’. This primer amplified the part Cyt b gene segment, followed TrnAlhr gene, the Pro tRNA gene and the D-loop segment.

The composition of the 25 µl PCR reaction was a DNA sample of 2 µl (10-100 ng), 1.25 units of RBC Biosciences Taq polymerase and its buffer system, 1 µL of 10 nmol of dNTP, 2 µl of MgCl2, 1 µl of AF22 and AF23 primers respectively, and DW sterile. All of the ingredients are combined into the PCR tube and then centrifuged at 3000 rpm for 30 seconds. The centrifuged material is put into the TAKARA MP4 Thermal Cycler machine for the amplification process.

The PCR conditions used for the mtDNA amplification process were the initial denaturation stage at 94°C for 3 minutes, the denaturation stage at 94°C for 45 seconds, the primary attachment stage (annealing) at 58°C for 30 seconds, and the polymerization stage (extension) at 72°C for 1 minute repeated for 30 cycles. The PCR reaction was terminated by polymerization (final extension) at 72°C for 5 minutes.

The visualization of PCR products was carried out using 6% polyacrylamide gel electrophoresis (PAGE) in a 1x TBE buffer. After that, it continued with sensitive silver staining (Tegelstrom, 1986) with a slight modification.

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DNA sequencing

The amplified DNA that showed a single band was then purified and molded in a PCR reaction for nucleotide tracing processes. The PCR reaction was carried out using the dideoxy terminator method with labeled dNTP (big dye terminator). Nucleotide tracing uses an engine branded ABI Prism 3700-Avant Genetic Analyzer.

Data analysis

The nucleotide sequences obtained are then aligned with the DNA sequences of several Bovidae groups that have been published in GenBank (http://ncbi.nlm.nih.gov). Data taken include B. javanicus 1 (FJ565566), B. javanicus 2 (EU878389), B. javanicus 3 (EF693809), B. taurus 1 (EU177815), B. taurus 2 (Friesian Holstein) (DQ124416), B taurus 3 (Beef cattle) (DQ124402), B. indicus (AF492350), Bubalusbubalus (AY702618). The alignment process using the Clustal W version 1.8 program embedded in the MEGA 4.0 program (Tamura et al., 2007). Reconstruction of phylogeny trees is based on the Kimura 2 parameter substitution model (K2P).

Reconstruction of phylogeny trees is based on the Cyt b and D-loop segments for all parsimony nucleotides. Reconstruction of phylogeny trees is carried out using the Neighbor Joining (NJ) method with 1000 times the bootstrap.

Results and Discussion

The total length of PCR products using the primary pairs AF22 and AF23 in Madura cattle ranges from 900-1300 (Figure 2). After BLAST and alignment process this primer produce 1396 bp amplikon, consist 203 bp Cyt b gene segment, followed by the 70 bp TrnaThr gene, 66 bp Pro tRNA gene and the D-loop segment of varying length 577 bp for the sample 41 and 29 sample. 624 bp for sample 32 (Figure 3).

The D-loop segment of the Madura cattle sample has various lengths. The variations are due to the deletion and insertion process. In D-loop sequence of the Madura cattle were found 22 nucleotide which experienced a repeated segments (tandem repeat). The recurring motive

Figure 1. Sampling location in 3 different area in Sampang regency, Madura Island (Googlemap, 2021).

Figure 2. Mitochondrial DNA band pattern amplified in PAGE 6% after silver staining. Column M is a 100 bp DNA marker, and the numbers in the next column refer to the Madura cattle sample number.
that appears is GTACATAATTA ATGTAAT. In the genus Bos, the recurring segment always begins with the GTAAT motif. Repeated segments were found in all sample of Madura cattle used. In Madura sample number 14, 26, and 38 repeated only once. The repeated sections in the sample, Madura cattle sample number 41, Madura cattle sample number 29, were repeated nine times and in the Madura cattle sample number 32 repeated three times (Figure 4).

Pessole et al. (1998) stated that the number of repetitions and the motive for repeating segments can differ between individuals and species. Repeating segments are not shared by all mammal species. Repeating segments with different motives were found by Nijman et al. (2003) on a sample of Madura cattle with the sequence ATTACATTAATATTATGTACTT that was repeated twice. In the family of bovidae, recurring segments have been documented in Ovis aries 75-76 nt in the D-loop segment (Hiendleder et al., 2002). The function of the repeating segments in D-loop is not certain. The existence of repeating segments tends to reduce the size of the genome in its evolutionary history (Avise, 1994). Repeating segments also form a strong coil rod structure (hairpin), this structure can inhibit the polymerase enzyme in vitro (Gemmel et al., 1996; Farajallah, 2005). It is thought that the structure of the coiled rod is one of the factors causing the low success rate during the amplification process.

The results of the phylogeny tree reconstruction using two mtDNA segments both stable (Cyt b) and those with high mutation rates (D-loop), showed Madura cattle samples divided into two groups in different branches. Madura cattle clade I (sample number 26, 14, and 38) are seen grouped in one branch with B. indicus. Madura cattle clade II (Samples number 29, 41, and 32) grouped with B. javanicus (Figure 4). The data obtained supports the results of the study of Nijman et al. (2003) which states that there are two types of mtDNA Madura cattle based on the D-loop segment, namely the type mtDNA B. indicus and the type mtDNA B. javanicus. This shows that Madura cattle are from two different ancestors of female breeders.

The small value of genetic distance based on both the Cyt b and D-loop genes shows that there is a close level of kinship between Madura cattle clade I with B. indicus and Madura cattle clade II groups with B. javanicus (Figure 5). These two groups are distinguished by 18 mutation points. The phylogeny tree using the base sequence and amino acid Cyt b shows the same topology. Madura cattle are divided into two groups. Madura cattle clade I (Samples number 26, 14, 38) were grouped in one branch with B. indicus with a bootstrap value of 98% for phylogeny trees based on amino acids (Figure 6A) and 96% for phylogeny trees based on base order (Figure 6B). Madura cattle clade I and B. indicus have the same nucleotide sequence in the segment Cyt b. Madura cattle clade II (Samples number 29, 41, 32) grouped with B. javanicus with a bootstrap value of 63% for phylogeny trees based on amino acids (Figure 6A) and 70% for base order (Figure 6B).

Figure 3. Organization of compilation of mitochondrial genes in a segment flanked by primary 22 and primary 23.

Figure 4. Some sections repeat tandem over 22 nt. Remarks in D-loop segment of Madura cattle (Mdr 29, Mdr 41, Mdr 32, Mdr 14, Mdr 26, Mdr 38), the first three lines indicate the nucleotide position number read vertically.
Figure 5. The value of genetic distance (below the diagonal) and the ratio of the incidence of transition and conversion (above the diagonal) were based on the Cyt b segment using the Kimura 2 Parameter (K2P) method.

Figure 6. Reconstruction of phylogeny trees based on nucleotide sequences (A) and Amino acid (B).

The grouping between clade II Madura cattle and B. javanicus was supported by a genetic distance value of 0.062 based on the Kimura 2 parameter model (Figure 5). The appearance of two female ancestors is probably due to the small success rate of the crossing between B. javanicus and B. indicus. The crossing is done in two ways, by using a B. javanicus male with female B. indicus or male B. indicus with female B. javanicus to obtain a fertile offspring.

According to Rollinson (1984) the small success rate of the crossing between B. indicus and B. javanicus is due to differences in the shape of the Y chromosome. Bos indicus has an Y chromosome that is acrocentric while B. javanicus has a metacentric Y chromosome. Differences in chromosome form result in disruption in the process of spermatogenesis, so that sometimes the resulting F1 male is sterile. Vietmeyer (1983) states that 1 of 4 females and 3 of 4 males from B. javanicus with B. indicus are sterile. The success of the cross between B. indicus and B. javanicus is 70%.

In the D-loop section the smallest genetic distance value based on the Kimura 2 model parameter was found between Bos javanicus and Madura 41 with a value of 0.000. Bubalus bubalus with Bos taurus (Friesian Holstein) had the largest genetic distance value of 0.293, with a ratio of the incidence of transition and conversion of 56/33 (Figure 7). The occurrence of substitution, both transition and tranversion in the D-loop segment, was more common than the Cyt b. This indicates that the D-loop segment is a segment that has a higher mutation rate.

The phylogeny topology based on the D-loop section is the same as the topology based on the Cyt b, that is, there are two groupings of Madura cattle. Madura cattle clade I grouped with B. indicus with 91% bootstrap and Madura cattle clade II with B. javanicus with bootstrap 96% (Figure 8). The crossing between B. javanicus and B. indicus was then more intensively carried out at the time of the government’s promoting the development of Ongole cattle (B. indicus) in the days of the Dutch East Indies. Ongole cattle (B. indicus) began to be brought to Sumba from Madras India in 1906. Furthermore, in 1915, 1919 and 1929 the breeds of cattle were distributed to several parts of Indonesia, especially Java. The descendants of the ongole cattle that have been distributed are then crossed with local beef cattle. The aim of the government to issue this policy is to create a nation of good quality beef cattle (Dwiyanto, 2008).
Figure 7. The value of genetic distance (below the diagonal) and the ratio of the incidence to transition to transformation (above the diagonal) based on the D-loop segment using the Kimura 2 Parameter (K2P) method.

Figure 8. Results of reconstruction of phylogeny trees based on the Dloop section using the NJ method with 1000x bootstrap.

The period from the beginning of the entry of the Indian nation to Indonesia and development of Ongol cattle to date is enough to form a stable Madura cattle nation. The formation of a stable cattle nation takes a long time around 10-20 years under intensive human control, for example by the method of artificial insemination. In natural conditions without human intervention, the formation of cows takes even longer, which is around 100 years (Simm, 2000). If generation time for cattle is 4-5 years (Dakay et al., 2006), it will take more than 25 generations to form a stable new cattle nation. Madura cattle already has these conditions and are classified as stable cattle.

The small value of genetic distance between Madura cattle clade I and B. indicus and Madura cattle clade II with B. javanicus shows the close level of kinship between these groups. The kinship distance between Madura cattle and B. taurus, B. indicus, and B. javanicus has been revealed in the research of Surjoatmodjo (1993) by comparing the morphological characters of B. taurus, B. indicus, Bali cattle and Madura cattle. Morphological characteristics were compared including gumba height (hump), body length, chest width, pelvic height, pelvic width, thigh width, chest circumference, forehead width, and forehead length. Based on the analysis of variants of the morphological characters, it was concluded that the closest Madurese cattle kinship distance was with the ongole breed (B. indicus) and the farthest from B. taurus. The distance value of Madurese cow kinship with Bali cattle is in the middle of the kinship distance value between B. taurus and Ongole breeds.

Conclusions

Based on the maternal lineage, Madurese cattle can be grouped into two types, type I originating from B. indicus and type II originating from B. javanicus. The results that allow the phylogeny tree to use the nucleotide data of the Cyt b gene and the Dloop segment of the mitochondrial genome determine how Madura type I cattle form a group with B. Indicus.

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Nirmala Fitria Firdhausi et al.  
Phylogenetic Study of Madura Cattle Based on Mitochondrial Cyt b

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