The mitochondrial DNA D-loop diversity of Bali cattle in breeding centers

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Abstract. Bali cattle is one important native cattle breeds of Indonesia which are contributing to the development of livestock and devote meat production. The aim of this study was to evaluate the genetic profiles using of mtDNA D-loop method in Breeding centers Bali cattle which consist BPTU Pulukan (Bali), BPTHMT Serading (West Nusa Tenggara) and VBC Barru district (South Sulawesi). The complete D-loop sequences, 1145bp in length, 48 individual sample from three breeding centers (24 males and 24 females) were analyzed. mtDNA D-loop amplification were performed by Polymerase Chain Reaction (PCR). The Molecular data of the D-loop mtDNA sequences were aligned and analyzed with reference (\textit{Bos indicus} and \textit{Bos taurus}) from GenBank using Basic Local Alignment Search Tool (BLAST) and MEGA version 5.0 software. The analyzed sequences length of the D-loop mtDNA was performed on 410 bp. The result of the mtDNA D-loop showed that there were 84 site variation were 2 site deletion, 8 site insertion and 74 site substitution of mtDNA D-loop Bali cattle. The nucleotide sequences of Bali cattle had a common Bali cattle in BPTU to BPTHMT with genetic distance more closely than the Bali cattle of VBC. It can be concluded that D-loop mtDNA could be used as potential candidate marker to differentiate and grouping in breeding centers of Bali cattle.

1. Introduction

Conceptually sustainable development is not only development in agriculture but holistically covers aspects of fisheries, forestry and animal husbandry. The economic approach in terms of sustainable agricultural policies, especially in the field of animal husbandry can be carried out with efforts to optimize and efficiently use scarce resources, which are imperative in dealing with various uncertainties. Management of resources by paying attention to their preservation is very important. One of Indonesia's livestock resources is Bali cattle. Bali cattle are the result of domestication of Banteng (\textit{Bibos banteng}) \cite{1,2} occurred before 3500 BC, allegedly occurring in Southeast Asia region and concentrated in Indonesia \cite{3}.

Bali cattle are developed, utilized and conserved as indigenous livestock resources that have specific characteristics and the ability to thrive in various environments that exist in Indonesia by displaying the performance of production is quite varied, and the performance of reproduction remains high. Thus, the Bali cattle genetic resources is one of the national asset, which is the germplasm needs to be protected...
and sustainably used because it has specific advantages. Bali cattle have also been included in cattle breeds which are listed in the FAO list as one of the world's existing breeds [4].

Bali cattle are able to adapt well and are very suitable to be developed by farmers as a national food source, this can be seen from the high population of Bali cattle in Indonesia [5]. This is the basis for the need for in-depth studies to obtain complete information in the preservation process. Efforts to maintain the unique characteristics of Bali cattle can be utilized in the future as one of Indonesia's superior cattle. However, the genetic quality of Bali cattle currently tends to decrease due to the lack of directed breeding efforts.

The D-loop region, in particular, has been used very widely, mtDNA D-loop region determine variations in mitochondrial DNA and the evolution of Japanese Black cattle (*Bos taurus*) [6], Position the Zebu cattle [7] originated from *Bos indicus* [8], the identification polymorphism of Benggala cattle, determine genetic differences and variations in the D-loop region sequences mtDNA Chinese native cattle[9], polymorphism of mtDNA D-loop on Philippine native cattle (*Bos indicus* and *Bos taurus*)[10], knowing hybridization Banteng (*Bos sondaicus*) and Zebu (*Bos indicus*) [11] and know the characteristics of Bali cattle [12]. Therefore, this research needs to be done, considering the Bali cattle genetic resources owned by Indonesia.

The result expected to provide necessary information about the diversity of genotype based on analyzed the mitochondrial DNA D-loop on Bali cattle in breeding centers which in this case represented by Bali, West Nusa Tenggara and South Sulawesi. This method using for very high degree of accuracy in describing the genetic diversity that results of this research as an information base for decision making in order to implement a more informed decision and directed the program of Bali cattle germplasm conservation, development and sustainable use.

2. Methods

2.1. Research time and location
This research base on the previous research on phenotypic diversity of Bali cattle. The study conducted through the identification of genetic diversity based on the diversity of mtDNA D-loop to confirm previous research. Research conducted at the Laboratory of Animal Molecular Genetics, Animal Breeding and Genetics Section, Department of Animal Production and Technology, IPB University.

2.2. Research materials

2.2.1. Animal samples. Bali cattle were taken from three populations in Superior Livestock Breeding Center (BPTU) of Pulukan of Bali province, Animal Breeding and Forage Feed Center (BPT-HMT) of Serading Sumbawa district of West Nusa Tenggara and the Village Breeding Centers (VBC) of Barru district South Sulawesi. The amount of each sample used for the diversity of mtDNA D-loop presented in table 1.

| Population                      | Male | Female |
|---------------------------------|------|--------|
| Bali cattle of BPTU Pulukan (Bali) | 8    | 8      |
| Bali cattle of BPTHMT Serading (West Nusa Tenggara) | 8    | 8      |
| Bali cattle of VBC Barru district (South Sulawesi) | 8    | 8      |
| Total                           | 24   | 24     |

2.2.2. Molecular approach. The materials used in blood sampling of cattle that tube, tube, box of ice (icebox), cotton and absolute alcohol. D-loop region amplification done through the Polymerase Chain Reaction (PCR). The materials used in the process of DNA amplification are DW, DNA samples, 2x Mastermix Kappa 2G Fast Ready Mix, forward and reverse primer. The tool used is a set of micropipette,
centrifuges, PCR tubes, PCR machines, vortex, and freezer. Materials used for electrophoresis consists of gel electrophoresis and 100 bp marker. The materials used to make the gel electrophoresis is agarose, 0.5 × TBE, Ethidium Bromide. Meanwhile, the tools used include microwave, steerer, magnets steerer, beakers, Erlenmeyer tube, gel tray, the printer for wells (comb), a power supply of 100 volts, measuring cups, macro and micropipette, as well as UV-Transilluminator. Buffer electrodes used consisted of Tris, Glycine and aquadestilata.

2.2.3. PCR amplification. A genetic analysis done with DNA isolation, amplification of the D-loop region of mitochondrial, electrophoresis of PCR product and sequencing of mitochondrial DNA D-loop. Before the isolation of DNA, blood which has preserved with absolute ethanol washed with Tris-EDTA low concentrations (lowTE). At each washing (after the addition of low TE) was centrifuged at 3000 rpm for two minutes and then repeated five times to eliminate absolute ethanol content contained in blood sample that has preserved with absolute ethanol. Isolation of total DNA using themethod of phenol-chloroform [13].

The amplification D-loop region was performed using machines Polymerase Chain Reaction (PCR) with the primers using software Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) based sequence D-loop region of mitochondrial DNA Bos indicus (Nollore cattle) accessible at NCBI GenBank (http://www.ncbi.nlm.nih.gov/) the access code AY126697 [14]. The length of 1145bp product with forward primer sequences 5'-TAGTACTAATACCAACAGCC-3' and reverse primer 5'-AGGCATTTTCAGTGCCTTGC-3'. PCR reagent used is a DNA template, aquabidest, forward and reverse primer and 2x Mastermix Kapa2G Fast Mix with a total reaction volume of 50 μl, As for the composition of the material for a reaction volume of 50μl is 2μl samples of DNA, each Forward and Reverse primer 0.5 μl and 2x mastermix Kapa2G Readymix Fast 25 μl and the rest of deionized water 22 μl, Prior to the materials incorporated into the PCR machine, first played briefly (spin down) with a microcentrifuge. Conditions PCR machine that is run by three stages: denaturation at 95°C for 45 seconds, the stage of annealing (annealing) at 60°C for 1 minute, and phase polymerization (extension) at 72°C for one minute was repeated as many as 35 cycles, beginning with a temperature of 95°C for five minutes and ended with polymerase (final extension) at 72°C for five minutes.

Electrophoresis using a 1.5% agarose gel. Agarose gel made, then heated on a hot plate stirrer to boiling (the solution looks clear), ethidium bromide is then added as much as 2.5 μl (10 mg/mL), and allowed to cool briefly. Before freezing agarose gel is poured into the mould which has prepared, then put the comb and let it freeze. Before running the electrophoresis, the sample is introduced into each of the wells sampled, solution or 1x TBE buffer (1 M Tris, 0.9 M boric acid, 0.01M EDTA pH 8.0) sample volume is sufficient and ready to run. Electrophoresis uses elektorforesis run tool or migrated from the negative (cathode) to the positive (anode) with a current of 100 volts, 200 mA for 30 minutes. Electrophoresis results observed with the aid of UV light (200-400 nm wave) and the images stored on a floppy disk or printed.

Sequencing D-loop DNA in the mitochondria to do with the reading sequence. Analyzed sequence into the primary and essential tool in molecular biology because it can determine the nucleotide and amino acid composition of a gene, was also used to analyze the kinship and the evolution path [15]. PCR products Mitochondrial DNA D-loop region done by sequencing process 1st BASE sequencing DNA Sequencing Services do the process.

2.2.4. Data Analysis. Analysis Mitochondrial DNA D-loop using the program Molecular Evolutionary Genetics Analysis [16]. The results analyzed with the program sequence Bioedit Sequence Alignment Editor [17].

3. Results and discussion

3.1. Amplification D-loop region of Bali cattle mtDNA
Amplification D-loop region Mitochondrial DNA (mtDNA) in samples from Bali cattle of breeding centers performed using forward primer 5'-TAGTACTAATACCAACAGCC-3' and reverse primer 5'-AGGCATTTCAGTCCTTGCC-3' results of its design based on base sequences Bos indicus mitochondrial DNA (Nellore cattle). Optimal results fragments of mtDNA D-loop successfully performed using Applied Biosystems PCR machine at annealing conditions with a temperature of 60°C for 1 min, and the obtained PCR product with a length of 1145 bp (figure 1).

To amplification success of D-loop region, in particular, is determined by the condition of the genomic DNA primer in addition to factors PCR reagents and PCR machine is used. There are variations in the size of the amplified fragment, on the N4 (female Bali cattle of BPHMT) compared with the results of the other sample amplification (± 1145bp). Variations in the size of the amplified DNA fragment likely caused by the presence of insertion (insertion) bases.

The base on alignment of sequences the whole genome DNA mitochondrial Bos indicus (Brahman cattle, Nollore cattle, Ongole cattle, Sahiwal cattle), Bos taurus (Simmental cattle, Limousin cattle, Angus cattle, Red Angus cattle) from GenBank, DNA fragments Bali cattle are amplified size of 1145 bp consisting of 52 bp fragment gene Cyt B at position 1081 up to 1140 (15602-15654), 68 bp tRNAThr in position 1 to 68 (15659-15727), 67 bp tRNAPro in position 1 to 67 (15 727-15 794), 913 bp fragments whole D-loop region at positions 1 through 913 (15795-16341, 1-366), and the 41 bp fragment tRNAPhe on-base position to-1 to 40 (367-407). Illustration layout primer annealing at the D-loop region of mtDNA Bali cattle research contained in figure 2. After sequencing the PCR products from two
directions forward primers and reverse primers, sequences of ± 1145 bp were obtained and mtDNA D-loop sequences of 913 bp were obtained.

3.2. Determination of the D-loop region and nucleotide diversity of Bali cattle mtDNA
Analysis diversity of sequences of nucleotides in the D-loop region of mtDNA is done after the DNA sequences of some of Bali cattle Breeding Centers aligned with Clustal W. The primary references in the sequence of sequence nucleotide Bos indicus and Bos taurus from GenBank (access code AB079327, AY126697, AY378135, L27732, AY676855, JN817331, AY676858 and DQ520591). The result of the sequence alignment shows that the number of bases in the D-loop region of each individual who has long studied varied. That is because there are mutations that eliminate (deletion) and insert (insertion) several nucleotides in Bali cattle BPTU, BPTHMT and VBC that each numbers are not the same. After the comparison is known that the number of nucleotide bases, each sample of Bali cattle in breeding centerss with the reference (B. indicus and B. taurus) is different. This turn of event because some deletions and insertions of nucleotides in the study sample. If one site deletions and insertions of the comparison results, it is considered a sample of research have a deletion or insertion, which of deletions and insertions varies between individuals.

The alignment of nucleotide sequences of Bali cattle in BPTU, VBC and BPTHMT along 410 bp which can be analyzed by reference to the sequence of B. indicus and B. taurus from GenBank, then every nation has a mean studied composition different nucleotide bases (table 2).

| Breed                     | n  | T (U) | C     | A     | G     | A+T   | C+G   |
|---------------------------|----|-------|-------|-------|-------|-------|-------|
| Bos indicus (Brahman cattle) | 1  | 30.9  | 24.0  | 29.4  | 15.7  | 60.3  | 39.7  |
| Bos indicus (Ongole cattle)  | 1  | 30.6  | 24.3  | 30.6  | 14.5  | 61.2  | 38.8  |
| Bos indicus (Nollore cattle) | 1  | 30.4  | 24.5  | 30.6  | 14.5  | 61.0  | 39.0  |
| Bos indicus (Sahriwal cattle) | 1  | 30.4  | 24.5  | 30.6  | 14.5  | 61.0  | 39.0  |
| Bos taurus (Simmental cattle) | 1  | 31.6  | 23.3  | 29.4  | 15.7  | 61.0  | 39.0  |
| Bos taurus (Angus cattle)    | 1  | 31.4  | 23.5  | 29.2  | 15.9  | 60.9  | 39.1  |
| Bos taurus (Limousin cattle) | 1  | 31.1  | 23.8  | 29.4  | 15.7  | 60.5  | 39.5  |
| Bos taurus (Red Angus cattle) | 1  | 31.4  | 23.5  | 29.5  | 15.2  | 61.3  | 38.7  |
| Bos sundaicus (Bali cattle of BPTU) | 16 | 28.2  | 27.0  | 30.1  | 14.6  | 58.4  | 41.6  |
| Bos sundaicus (Bali cattle of BPTHMT) | 16 | 28.2  | 27.0  | 30.1  | 14.6  | 58.4  | 41.6  |
| Bos sundaicus (Bali cattle of VBC) | 16 | 28.2  | 27.0  | 30.2  | 14.6  | 58.3  | 41.7  |

The average of the highest nucleotide T at Bali cattle and the reference to the order began highest Brahman cattle (30.9), and the lowest is in Bali cattle research sample (28.2). Mean C nucleotide highest to the lowest row of Bali cattle shown in the study sample followed by Bos indicus and Bos taurus. The average of the highest nucleotide A Bos indicus and Bos taurus lows. Bali cattle samples had the lowest mean G nucleotides compared to Bos indicus and Bos taurus. In Bali cattle, nucleotide T (U), C and G D-loop region of mtDNA have the same mean frequency of several breeding centers namely 28.2, 27.0 and 14.6. A nucleotide was averaging the highest frequency in samples of Bali cattle research that Bali cattle VBC (30.2) (Figure 3). The diversity of nucleotide bases is due to mtDNA having a mutation rate of five to ten times faster than nuclear DNA [18] and in the control region have an evolutionary speed of 10-20 times faster than other mtDNA regions [19]. The difference of composition nucleotides in Bali cattle BPTU and BPTHMT with VBC are seen in nucleotides A (0.01%). The average composition between BPTU and BPTHMT to be similar. The existence of nucleotide differences between Bali cattle
from breeding centers with references (Bos indicus and Bos taurus from GenBank) indicate the existence of deletions and insertions.

Based on alignment is found that the composition of the nucleotides at the most construct mtDNA D-loop partial in this study are A and T, which amounted to 60.3% (Brahman cattle), 61.2% (Ongole cattle), 61% (Nellore cattle, Sahiwal cattle and Simmental cattle), 60.9% (Angus cattle), 60.5% (Limousin cattle), 61.3% (Red Angus cattle), 58.4% (Bali cattle of BPTU and BPTHMT) and 58.3% (Bali cattle of VBC) (table 2) (figure 3). Average composition of the nucleotide bases A+T has a higher frequency compared with composition G+C in this study because the area is a noncoding region. It suspected of cause of noncoding regions have a higher rate of evolution and closely related to the character of mtDNA D-loop that has many early sites of replication for heavy thread (heavy strand) mtDNA transcription and as a starting point for light weight thread (light strand) or heavy thread [20]. Pattern on the base composition of this study follows the mammals in general, where the composition of the largest bases in mitochondrial DNA are A and T [20] and the proportion of A and T nucleotides in the mitochondrial genome of cattle approximately 70% [21].

![Figure 3. Frequency nucleotide region of partial mtDNA D-loop size 410 bp on Bali cattle of breeding centers and reference (B. sundaicus, B. indicus and B. taurus) from GenBank](image)

The average composition of the nucleotide bases G+C on a sample of this study show that 41.6% (Bali cattle of BPTU and BPTHMT) and 41.7% (Bali cattle of VBC) is higher than the reference. At a temperature of thermophilic bacteria capable of living has a composition of a high G+C [22]. Due to the bond between G+C is more stable than the bond at the A+T so that Bali cattle have better endurance capability in tropical environments than B. taurus and B. indicus.

The comparison of amino acid composition which contains showed differences in nucleotide composition A, T, G, and C [23]. When compared to the average composition of the nucleotides between Bali cattle from breeding centers with reference to the average of the lowest composition of the differences are Ongole cattle, Nellore cattle and Sahiwal cattle. Profound differences mean nucleotide composition of Bali cattle of breeding centers with reference because Bali cattle derived from domesticated cattle, domestication banteng (Bos javanicus) [11] while the reference cattle from the Bos indicus and Bos taurus.
The result of nucleotide ratio bases in a sample size of 410nt in Bali cattle from breeding centers with reference (B. indicus and B. taurus) from GenBank, showed as many as 84 sites several sites of bases. The average value of the ratio of transitions (substitution of a purine or pyrimidine purine with a pyrimidine) to transversion (alteration of the purine to pyrimidine or vice versa) is 2% (purines) and 12% (pyrimidine) with the value of the overall bias (R) = 0.536. Overall there are 2 insertion sites, 8 deletions sites and 74 sites substitute for Bali cattle samples from breeding centers with reference Bos indicus (Brahman). [12] that states that in Aceh cattle against B. indicus have 9 sites different with the average value of the ratio of transition to transversion is 1.13% and 1.86% with a bias value 0.533 for overall. The diversity arrangement of nucleotide bases in the D-loop region of mtDNA samples Bali cattle this research indicate mutations. In mammals, mtDNA inheritance through the mother reported no recombination [19] and have a high degree of substitution [20] estimated to be 2-8.5 times higher than elsewhere in the mitochondria [6].

4. Conclusion

Bali cattle contained in different of breeding centerss in the D-loop mtDNA. The results analyzed of the mtDNA D-loop based on the sequence of nucleotide bases on various sites. The sequence of nucleotide bases Bali cattle of BPTU have genetic similarities that are closer to the Bali cattle of BPTHMT compared Bali cattle of VBC, so it can be concluded that D-loop mtDNA could be used as potential candidate marker to differentiate and grouping in breeding centerss of Bali cattle.

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