



Sequencing and Phylogenetics of the Cytochrom B Gene in Bali Cows With Different Coat Color in Kupang, East Nusa Tenggara, Indonesia

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Abstract | Bali cows are native Indonesian cattle reared by farms in Kupang with varying coat color. The research objective was to determine the Bali cows with different coat color in Kupang district based on DNA analysis using the cytochrome b (*CYT B*) gene. The methods used *CYT B* gene base methods of DNA analysis such as DNA extraction, Polymerase Chain Restriction, DNA sequencing, and identification of DNA sequences nucleotide. Bali cows with sorrel, black and white coat colors are used with an age range of 3-5 years. DNA sequencing conducted multiple alignments, the identification of the genetic distance, and the preparation of phylogenetic trees using the software Mega X. The results of PCR sequencing of Bali cows with different coat colors in Kupang were 464 bp. Identification of single nucleotide polymorphism in Bali cows compared to *Bos taurus* and *Bos javanicus* as many as 29 SNPs. The percentage of nucleotide similarity with *Bos taurus* was 93.75% and the nucleotide diversity was 6.25%. The percentage of nucleotide similarity with *Bos javanicus* is 100%. The conclusion is that Bali cows with different coat colors in Kupang have 100% genetic similarity with *Bos javanicus* and the *CYT B* gene can be used as a genetic marker for Bali cows with different coat colors in Kupang.

Keywords | Bali cows, Coat color, Cytochrome b, Sequencing and phylogenetic tree.

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INTRODUCTION

Deviation of coat color from sorrel coat color by 17%, namely the color of white socks mixed with brick red/brown/black, striped Bali cattle (0.6%) and black (0.3%) (Handiwirawan and Subandriyo 2004). The allele frequency of Melanocortin 1 receptor (MC1R) gene in Bali cows with different coat colors in kupang is 99% monomorphic and 1% polymorphic (Tabun et al., 2013), percentage of nucleotide similarity were 99.662% at black Bali cow, Sorrel and white Bali cows were 98.986% with *Bos taurus* (Tabun et al., 2020).

Livestock mating systems can also influence color differences that can increase the pressure of inbreeding. Extensive and semi-intensive cattle rearing system in Kupang has led to mating between Bali cattle that still have genetic relationships. Mohamad et al. (2012), stated that the high rate of *inbreeding* among Bali cows in several locations in Indonesia, namely the islands of Bali, Sulawesi and Sumatra has a closer genetic distance to the bull and lower levels of variability.

Deviations in coat color in Bali cows require research to determine the Bali cows with different coat color in Kupang Regency using the *CYT B* gene. Syed-Shabthar et al.

(2013), identification of livestock kinship relationships can make historical sketches, origins of cattle which can assist in the development of livestock breeding and conservation programs. Mohamad *et al.* (2009), reveals the systematic relationship of Bali cows using mitochondrial DNA, Y chromosome and microsatellites. Rosli *et al.* (2011), state that mitochondrial genome used for phylogenetic studies. The *CYT B* gene was used in the investigation of systematic relationships between mammals. This is due to the higher variation of the *CYT B* gene compared to that of other functional region, the fact that it is easily detected and the availability of high-definition phylogenetic information, especially for *species-level* classification. The *CYT B* gene is an indicator of phylogenetic relationships among species used as a tool to identify relationships between species (Mohamad *et al.*, 2009).

Differences in coat color in female Bali cattle in Kupang, caused by crossing or the effect of inbreeding pressure, make to research to identification of Bali cows with different coat color in Kupang, so that are to provide a further help in the study of the pedigree and evolution of Bali cows, and it will provide a great help for the protection of genetic resources

MATERIALS AND METHODS

RESEARCH SAMPLES

Blood samples were taken from the jugular venous of Bali cows with different coat color in Kupang were 59 cattles. Blood samples consisted of 34 samples from sorrel Bali cows, 17 samples from black Bali cows and 8 samples from white Bali cows.

DNA EXTRACTION

DNA extraction from blood samples of Bali cows with different coat colors using a modified manual protocol for DNA isolation of blood samples by Sambrook *et al.* (1989). The results of DNA extraction from 59 blood samples were electrophoresed horizontally using 2% agarose. The analysis of DNA samples was conducted on the Laboratory of Animal Breeding, Faculty of Animal Science, Gadjah Mada University, Indonesia.

POLYMERASE CHAIN REACTION (PCR)

Amplification of the *CYT B* gene used primers was L14735: 5'-AAAAACCCGTT GTTATTCAACT-3' and H15149: 5'-GCCCCTCAGA ATGATATTTGTCCTCA-3'. The DNA amplification process was carried out with a total volume of 10 µl consist of 0.5 µl DNA, 5 µl PCR Mix, 1 µl 10 Pmol primer and 3.5 µl *Double Distilled Water* (DDW). PCR amplification of the *CYT B* gene of 464 bp using a Thermal Cycler machine with *pre denaturation* conditions at 94 °C for 2 minutes, *denaturation* at

95°C for 36 seconds, *annealing* at 51 °C for 73 seconds and *extension* at 72 °C for 84 seconds repeated 35 cycles, and *final extension* at 72 °C for 3 minutes.

DNA SEQUENCING

CYT B gene sequencing can identify the nucleotide sequence of Bali cows with different coat color in Kupang. The *CYT B* gene PCR product was purified, then used as template DNA for the DNA sequencing reaction. The total volume of the MC1R gene amplification reactions was 30 µl consist of 1.5 µl DNA, 15 µl PCR Mix (Kappa Mix), 1.5 µl 10 pmol L14735 primer, 1.5 µl 10 pmol H15149 primer and 10.5 µl DDW. The results of DNA sequencing were carried out by multiple alignments, single nucleotide polymorphism (SNP) identification and phylogenetic trees using *MegaX software*.

RESULTS AND DISCUSSIONS

PCR APLIFICATION OF THE *CYT B* GENE

Amplification of the *CYT B* gene in Bali cows with different coat colors in Kupang using primers L14735 and H15149 in accordance with the instruction (Hartatik *et al.*, 2015). The primer pair amplifies the DNA fragment by 464 *base pairs* (bp). *CYT B* gene PCR products in Bali cows with different coat colors in Kupang can be seen at Figure 1.

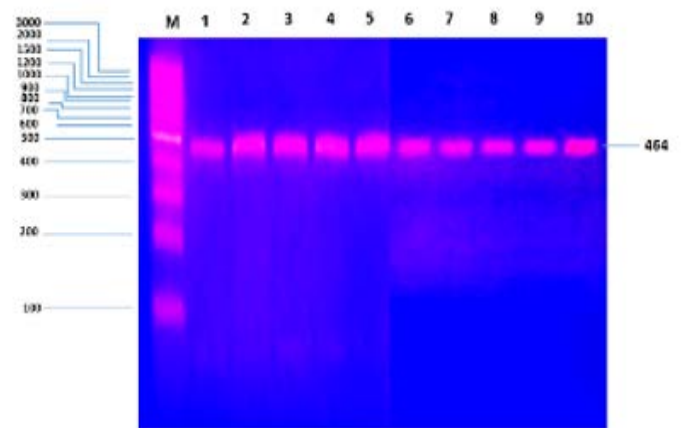


Figure 1: PCR products of *CYT B* gene of Sorrel, black, and white Bali cows. Lane M: DNA marker ladder, Lane 1, 2, 3, 4, 5, 6, 7, 8, 9, 10.

CYT B GENE SEQUENCING

Multi alignments nucleotide *CYT B* gene of sorrel, black and white Bali cows with standard gene from NCBI namely *CYT B* gene complete from *Bos taurus* (DQ186224), *Bos Indicus* (JN817305.1; JN817299.1; AY126697.1; AF492350.1) and *Bos javanicus* (AB915322.1; D34636.1). The *CYT B* gene of Bali cows in Kupang can be seen at Figure 2.

Table 1: The percentage nucleotides of similarity and diversity of *CYT B* gene of Bali cows with different coat color in Kupang compared to *Bos taurus* (DQ186224.1), *Bos indicus* and *Bos javanicus* (AB915322.1 and D34636.1).

Types of Cows	N	Number of nucleotides	Number of nucleotides in common	Percentage of nucleotide similarities (%)	Number of nucleotide diversity (SNP)	Percentage of nucleotide diversity (%)
BOS_TAURUS_DQ186224		464	464	100.00	0	0.00
BOS_INDICUS_JN817305.1		464	464	100.00	0	0.00
BOS_INDICUS_JN817299.1		464	464	100.00	0	0.00
BOS_INDICUS_AY126697.1		464	463	99.78	1	0.22
BOS_INDICUS_AY126697.2		464	463	99.78	1	0.22
BOS_INDICUS_AF492350.1		464	463	99.78	1	0.22
BOS_JAVANICUS_AB915322.1		464	435	93.75	29	6.25
BOS_JAVANICUS_D34636.1		464	435	93.75	29	6.25
SORREL BALI COWS	34	464	435	93.75	29	6.25
BLACK BALI COWS	17	464	435	93.75	29	6.25
WHITE BALI COWS	8	464	435	93.75	29	6.25
TOTAL	59					
AVERAGE				94.29		5.72
STANDARD DEVIATION				1.74		1.74

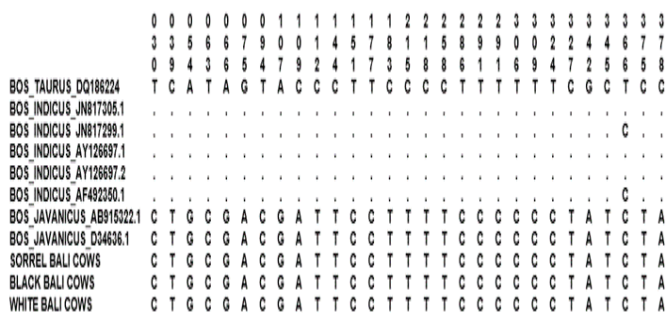


Figure 2: The results of sequencing nucleotide of *CYT B* gene Bali cows are different coat colors with *Bos Taurus*, *Bos Indicus* and *Bos javanicus*.

GENETIC DISTANCE

Genetic distance analysis of Bali cows using Pearson (*Mega10*) method shows that the genetic distance between Bali cows with different coat color in Kupang compared to NCBI *GenBank* standart namely *Bos taurus*, *Bos indicus* and *Bos javanicus* can be seen at [Table 1](#).

PHYLOGENETIC TREE

The result of sequencing *CYT B* gene of Bali cows with different coat colors in Kupang compare *Bos taurus*, *Bos indicus* and *Bos javanicus* standard gene sequence from NCBI-*GenBank* with accession gene number: D34636.1. The results of the phylogenetic tree analysis on *Mega X* (*Rzhetsky and Nei, 1992; Saitou and Nei, 1987; Nei and Kumar, 2000*), can be seen in [Figure 3](#).

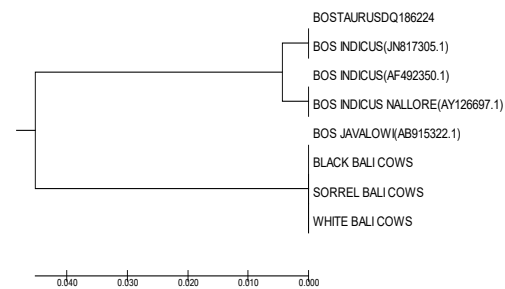


Figure 3: Bali cows phylogenetic tree with different coat colors in Kupang

DISCUSSION

CYT B gene in Bali cows with different coat colors in Kupang compared to *Bos taurus*, *Bos indicus* and *Bos javanicus* used *MegaX* software program. This shows that the genetic similarity with *Bos taurus* and *Bos indicus* is 93.75% and the nucleotide diversity is 6.25%. The results of multiple alignments of Bali cows with different coat colors in Kupang compare to *Bos javanicus* showed 100% nucleotide similarity. [Rahmatullaili \(2010\)](#) states that genetically, the population of Bali cows in the territory Bali and Lombok region can be grouped into 7 large groups. the closest genetic distance between The Bali lombok cow and the Bull (*Bos javanicus*) is 0.061 while the coat the distance between Bali Lombok 8 and *Banteng* is 0.312. [Rosli et al. \(2011\)](#) states that the genetic similarities between Bali bulls and cows and their proximity to Malayan gaur cows. The genetic

distance between Bull and Bali cows compared to Malayan gaur cattle has similarities. While domestic zebu and taurine cattle have a greater genetic distance with Malayan gaur.

The results of *multiple alignment* using *Mega X software* found changes in nucleotide sequences of *CYT B* gene Bali cows with different coat colors in Kupang. Nucleotide changes in the order with single nucleotide polymorphism (SNP) were found. Changes in the composition of nucleotides in some squamates are called nonsense mutations, which are the process of changing codon from amino acid bases but do not cause protein formation errors. This change does not cause changes in amino acids from the formed codon. Change in the composition of nucleotides in c.75G/A where a change of one nucleotide in the codon causes a change in amino acids from *Tryptophan* to *Stop*. Changes in nucleotides in sequence 75 cause a *point mutation* that causes the change of an alkaline pair where one base on one DNA sequence is replaced with a different base. This mutation can cause several things depending on where the mutation is located in the gene. If alkaline replacement takes place within the gene that encodes the protein, then the mRNA transcription of the gene will carry the wrong base. Mitochondria RNA is translated into proteins, such alkaline errors can cause the formation of proteins, or the formation of abnormal proteins, or the formation of nonsense codon (codon STOP) that stops the complete synthesis of functional proteins (*nonsense mutations*). Changes in nucleotides c107A/G and c109C/A caused changes in two nucleotides in one protein codon causing changes in the amino acid *Isoleucine* to *Valin*. Nucleotide changes from c151T/C where there are change in the serine to proline.

The nucleotide sequences of Bali cows with different coat colors in Kupang (Table 1) compared to the *Bos taurus* sequence (DQ186224.1) showed that the percentage of nucleotide similarity was 93.75% and the nucleotide diversity was 6.25%. The nucleotide sequences of Bali cows with sorrel, black and white coat colors compared to the *Bos javanicus* standart sequence have 100% nucleotide similarity. This shows that Bali cows with different coat colors in Kupang have a genetic relationship with *Bos javanicus*. Bali cattle on three different islands in Indonesia have the same mitochondrial DNA sequence as the bison (Mohamad et al. 2009). Haplotype 1 and haplotype 2 of Bali cattle mitochondrial DNA sequences are almost the same as the Bison. The D-loop mitochondrial DNA sequence of Bali cattle are different from the D-loop sequence of *Bos taurus*, *Bos indicus*, *Bos javanicus*, and *Bos gaurus* (Wisesa et al., 2012). In this study, it is different from the result of research of Bali cattle in Malaysia, which are almost identical to Sebu cattle. The sequence of bali cattle in Malaysia is almost identical (99.5%) to zebu cattle. Meanwhile, Ma-

dura cattle are almost identical (99.7%) with bulls (Nijman et al. 2003).

In Figure 3, the phylogenic trees of Bali cows with sorrel, black and white coat colors in Kupang are compared with the standard gene sequences of *Bos taurus*, *Bos indicus*, *Bos javanicus* from the *National Center for Biotechnology Information* (NCBI)-*GenBank* with gene accession number: D34636.1 which has a 100% kinship relationship. Margawati et al. (2017), stated that the high sequence of nucleotide similarities from Bali cattle at each location indicated that there was lower variability among Bali cattle due to inbreeding. Rahmatullaili et al. (2019), states that the phylogenetic trees of Bali cattle in Bali and Lombok regions with the *CYT B* gene can be grouped into 7 major groups. The closest genetic distance between Bali Cattle in Lombok and *Bos javanicus* are 0.061 while the farthest distance between Bali Lombok cattle and *bison* are 0.312.

The genetic distance between Indonesian cattle and other cattle breeds ranged from 0.002 to 0.077 (Tamura et al., 2004; Kumar et al., 2018). The estimated genetic distances was the closest between Indonesian cattle and *B. javanicus* with a value of 0.002 (Hartatik et al., 2019). Septian et al. (2015), reported that the genetic distance of Bali cattle from superior livestock breeding center Bali cattle, Barru Village Breeding center, South Sulawesi and livestock breeding center - forage fodder Seranding-Sumbawa (West Nusa Tenggara) with microsatellites at INR037 and ETH185 had a variability of 0.033%. Bali cattle from Riau, South Kalimantan, West Nusa Tenggara (Bima and Mataram) and Nusa Penida (Bali) were in the same group to *bison* and *Bos javanicus*. However, it was different from bison group. The early finding can be used either for conservation decision or for future breeding strategy of Bali cattle (Margawati et al., 2017).

CONCLUSION

The *Cyt B* gene sequence in Bali cows with sorrel, black and white coat colors in Kupang has 100% nucleotide similarity with *Bos javanicus*, 93.75% nucleotide similarity and 6.25% nucleotide diversity with *Bos taurus*. The *CYT B* gene can be used as a genetic marker for Bali cows with different coat colors in Kupang.

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CONFLICT OF INTEREST

The authors declared they have no conflict of interest.

NOVELTY STATEMENT

Research novelty is to find the kinship relationship (cyt b gene) of Bali cows with different coat colors in Kupang with the Bali cattle and banteng breeds.

AUTHORS' CONTRIBUTION

ACT developed a joint research plan with FSS, CLP, JAJ and TL. The research passed selection at Ministry of Technology Research and Higher Education, all the time involved in carrying out field work, collecting field research data. ACT conducted DNA analysis work in the laboratory and collected the manual then corrected by CLP, JAJ, FSS, TH and approved the contents of the manuscript.

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