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Investigating genetic diversity of Indonesian native cattle breeds using mitochondrial DNA 16S rRNA gene

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Abstract: This study aimed to estimate the genetic diversity and phylogenetic relationships of Indonesian native and local cattle breeds using 16S rRNA mitochondrial DNA. The study analyzed 93 DNA samples from six cattle breeds. The polymerase chain reaction was conducted using two primer pairs to sequence the complete 16S rRNA gene. The number of the polymorphic site (S), pairwise nucleotide differences (K), haplotype diversity (Hd), and nucleotide diversity (π) were estimated using DnaSP 6 software. PopART v. 1.7 software was employed for the median-joining network analysis, while a neighbor-joining tree was reconstructed based on the Kimura 2-parameter model using MEGA X software. The results revealed 29 haplotypes, 14 in Bali cattle. The haplotype diversity varied from 0.24 ± 0.135 to 0.92 ± 0.084. The overall nucleotide diversity among populations was 0.0190 ± 0.0011. Indonesian native cattle revealed high variability in the 16SrRNA gene sequence. The phylogenetic analysis assigned the Bali cattle as Indonesian native cattle separated from the other cattle breed.

Keywords: Bali cattle, 16S rRNA gene, mtDNA

1. Introduction

Indonesia has various native cattle breeds, including Bali cattle (*Bos javanicus*) and other breeds, such as Pesisir, Sumba Ongole, Madura, Aceh, Grati Ongole Grade, Kuantan, Sumbawa, Pasundan, Jabres, and Donggala, known as local Indonesian cattle. Some studies reported that there are three types of local cattle in Indonesia, which are zebu (*Bos indicus*), Bali cattle (*Bos javanicus*), and taurine cattle (*Bos taurus*) [1,2].

The cattle populations in Indonesia are very adaptive to the Indonesian people's environmental conditions and farming culture. Therefore, it is necessary to have a structured breeding program to preserve and develop Indonesian cattle. Mitochondria DNA (mtDNA), as a source of genetic information based on maternal inheritance, has been widely used in the study of population genetics and biodiversity because it enables essential input for phylogenetic analysis, evolutionary biology, identifying areas of domestication, maternal inheritance, and geographic origin of livestock [3,4]. Mitochondrial DNA consists of a displacement loop (D-loop) region and 37 genes (13 parts protein (polypeptides), 22 parts transfer RNA (tRNA), and two parts (small (12S) and large (16S)) ribosomal RNA (rRNA). To date, mitochondrial DNA sequence analysis has been applied to investigate Indonesian local cattle diversity, although partially carried out on D-loop and cytochrome b (cytb) genes. For instance, D-loop markers were applied in the analysis of mitochondrial DNA diversity on Bali cattle [5] and Aceh cattle [6]. Cytb gene has also been employed in the analysis of Bali, Donggala, Madura, Sragen, Galekan, Rambon, and POBA cattle [7–9].

In addition to mtNA D-loop and cytb genes, the 16S rRNA gene located on mtDNA may also serve as a genetic marker for detecting animal genetic diversity and phylogenetic studies. It was similarly employed in studies on Chinese cattle, Iranian Afshari sheep, Nili-Ravi buffalo, Khuzestan buffalo, family Bovidae (including Mountain goat, Ibex, bison, buffalo, antelopes, gazelles, sheep, goats, muskox, and domestic cattle) [10–15]. Although the use of 16S rRNA marker for genetic diversity and phylogenetic analyses in cattle appears in the literature, studies using

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16S rRNA complete sequence are still unavailable in Bali cattle and other Indonesian local cattle breeds (Pesisir, Pasundan, Ongole, Kuantan, and Madura). It is noteworthy that the 16S rRNA gene can be an advanced marker to characterize genetic diversity and phylogenetic analysis [10]. The use of molecular markers for genotyping and diversity assessment, including 16S rRNA, is essential in completing basic scientific data required for the development and conservation of Indonesian native and local cattle. Therefore, the objectives of this study were; to estimate the genetic diversity and to reveal the phylogenetic relationships within and among Indonesian native and local cattle breeds based on 16S rRNA mitochondrial DNA.

2. Materials and methods

2.1. DNA samples

A total of 93 DNA samples were analyzed, including Bali cattle from two subpopulations (UPT Bali and Nusa Penida) and five local Indonesian cattle breeds consisting of Kuantan, Pesisir, Madura, Peranakan Ongole (PO), and Pasundan cattle (Table 1). DNA samples belonged to a Genetic and Molecular Laboratory collection in IPB University and were collected from 2012–2021.

2.2. Primer, amplification, sequencing

The primer set included two pairs of primers (Table 2). The primer design was carried out using Primer 3 software (http://primer3.ut.ee) based on *Bos indicus* mitochondrial complete sequence (GenBank Accession No. AY126697).

The polymerase chain reaction (PCR) was conducted in a final volume of 25 µL consisting of 2 µL DNA sampel (100 ng), 12.5 µL GoTaq Green 2X Master Mix (Promega, United States), 0.25 µL of each primer forward and reverse (25 pmol/µL), and 10 µL nuclease-free water.

The PCR reactions were performed using Applied Biosystem Thermal Cycler under the following conditions: 1 cyle of predenaturation (95 °C for 5 min), 35 cycles of amplification (denaturation at 95 °C for 10 s, annealing at 57 °C for 20 s, extension at 72 °C for 30 s), and the final elongation at 72 °C for 5 min. The PCR products were visualized by agarose gel electrophoresis (1.5% agarose gel and 0.5xTBE buffer).

The complete 16S rRNA (1569 bp) region was successfully amplified and sequenced by two primer sets targeting two separate regions with an overlapping region for alignment purposes. The first primer pair (16S rRNA1) amplified the region between the 1038 and 1883 nucleotide positions, with a fragment length of 846 bp. The second primer set (16S rRNA2) amplified the region between the 1760 and 2728 nucleotide positions, with a fragment length of 969 bp (Figure 1). Then, for sequencing, the PCR fragments were sent to 1st BASE Laboratories, Selangor, Malaysia.

2.3. Data analysis

The 16S rRNA sequences were aligned using ClustalW implemented within the MEGA X software [16]. The

Table 1. Information on the six cattle breeds investigated in this study.

No	Breed	Description	Number of animal	Location
1	Bali cattle	Native cattle (*Bos javanicus*)	44	Breeding Center, Bali province
2	Kuantan cattle	Local cattle (Crossbred)	16	VBC Kuantan district, Riau Province
3	Pesisir cattle	Local cattle (Crossbred)	8	Breeding Center, Padang Mangatas district, West Sumatera
4	Madura cattle	Local cattle (Crossbred)	8	VBC Pamekasan district, East Java
5	PO cattle	Local cattle (*Bos indicus*)	9	VBC Kebumen district, Central Java
6	Pasundan cattle	Local cattle (Crossbred)	8	VBC Ciamis district, West Java

Table 2. Primer sequences employed for the amplification of 16S rRNA gene.

Primer set	Sequenced* (5'- 3')	Length of PCR product (bp)	Temperature annealing (°C)
16srRNA-1	Forward: AACAAA GCATCCAGTTTACACC	846	55
	Reverse: TTTCCTTAGATGCACTCCTGTG		
16srRNA-2	Forward: CCTTGCATAAGTCTAAGTCAGTGC	969 pb	57
	Reverse: GAGAGGATTTGAATCTCTGGGTA		

*GenBank access No. AY126697.

analyzed sequence diversity was expressed as the number of the polymorphic site (S), the pairwise nucleotide differences (K), haplotype diversity (Hd), and nucleotide diversity (π); and these parameters were estimated using DnaSP version 6.12.01 software [17]. The median-joining network [18] analysis was carried out by using PopART software [19]. The neighbor-joining tree was constructed based on Kimura 2-parameter model, with the following parameters: 1000 bootstrapping replicates, a gamma distribution (+G) with five rate categories, and evolutionary invariability (+I) [16]).

3. Results

3.1. Diversity of 16S rRNA mitochondrial DNA

The complete sequence of the mtDNA 16S sRNA (1569 bp) was obtained for all 93 samples. The base composition was as follows: 23.9% thymine, 20.8% cytosine, 37.4% adenine, and 17.9% guanine. The estimated diversity parameters based on 16S rRNA complete sequence in Bali and other Indonesian local cattle breeds were given in Table 3.

A total of 95 polymorphic sites and 29 haplotypes were observed in this study. The number of haplotypes found in breeds ranged from 2 to 14. In this regard, Bali cattle had the highest number of haplotypes. On the contrary, the lowest one was attributable to Pesisir cattle. The haplotype diversity varied from 0.24 ± 0.135 (Kuantan cattle) to 0.92 ± 0.084 (Pasundan cattle) (Table 3). The overall nucleotide diversity among populations was 0.0190 ± 0.0011.

The distribution and observed frequency of the haplotypes found across the breeds of the study was given in Table 4. There were 29 haplotypes obtained from 93 16S rRNA sequences. H1 became a major haplotype with 25 sequences, consisting of Bali cattle, Madura cattle, and Pasundan cattle. Furthermore, H15 was the second-largest haplotype, consisting of Kuantan cattle and Pesisir cattle. We also found some private haplotypes for specific breeds; such as H3-H14 only detected in Bali cattle, H16-H17 only found in Kuantan cattle, and H19 in Pesisir H21-H22 in Madura cattle, and H27-29 in Pasundan cattle.

3.2. Genetic distance and phylogenetic structure

A median-joining network was constructed to determine the phylogenetic relationship among the 29 haplotypes using popART program (Figure 2) [19]. The haplotypes were grouped into two main lineages (A and B). The Bali cattle were distributed in lineage A, while Pesisir, Kuantan, and PO were in lineage B. Although Madura and Pasundan

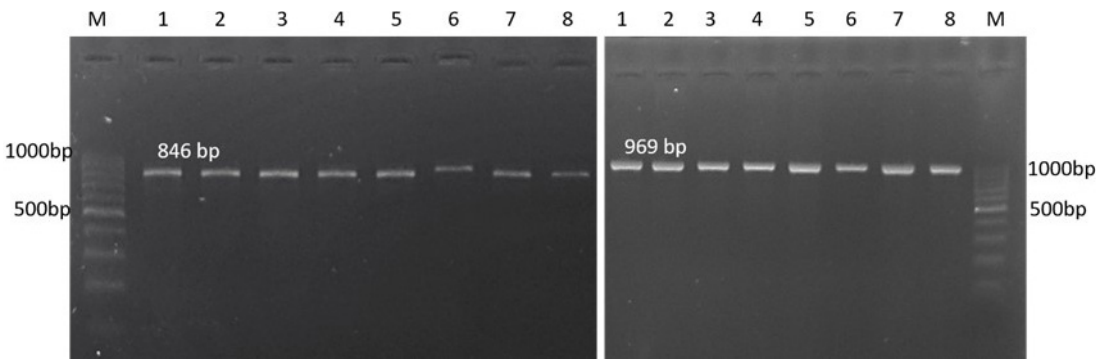

Figure 1. Amplification of 16S sRNA complete sequence (A: 16S sRNA1; B:16S sRNA2; M: 100 bp Marker; 1–8: Number of samples).

Table 3. Genetic diversity 16SrRNA complete sequence in Bali and Indonesian local cattle breeds.

Breeds	N	S	H	K	Hd	π
Bali	44	17	14	1.45	0.70 ± 0.063	0.0015 ± 0.0002
Kuantan	16	4	3	0.50	0.24 ± 0.135	0.0003 ± 0.0002
Pesisir	8	1	2	0.25	0.25 ± 0.180	0.0001 ± 0.0001
Madura	8	68	5	30.17	0.86 ± 0.108	0.0191 ± 0.0054
PO	9	66	7	31.00	0.81 ± 0.159	0.0225 ± 0.0052
Pasundan	8	72	6	30.85	0.92 ± 0.084	0.0192 ± 0.0058
Overall	93	95	35	30,13	0.86 ± 0.022	0.0190 ± 0.0011

N : Number of sample, S: Segregating Site, H: Number of Haplotype, K: Nucleotide Differences, Hd : Haplotype Diversities, Π: Nucleotide diversity.

Table 4. Haplotype distribution among Bali and Indonesian local cattle.

Haplotype	Breeds					
	Bali	Kuantan	Pesisir	Madura	PO	Pasundan
H1	22			2		1
H2	10				1	2
H3	1					
H4	1					
H5	1					
H6	1					
H7	1					
H8	1					
H9	1					
H10	1					
H11	1					
H12	1					
H13	1					
H14	1					
H15		14	7			
H16		1				
H17		1				
H18			1			
H19				1		
H20				3	3	2
H21				1		
H22				1		
H23					1	
H24					1	
H25					1	
H26					1	
H27					1	1
H28						1
H29						1

cattle were mainly distributed in lineage B, two samples from Madura and Pasundan breeds were in lineage A.

The result of genetic distance estimates revealed that Bali cattle and Pesisir cattle had the highest genetic distance; meanwhile, Pesisir and Kuantan cattle had the lowest genetic distance. Table 5 shows the pairwise genetic distance estimates among six Indonesian cattle breeds based on a 16S rRNA complete sequence. Furthermore, a neighbor-joining phylogenetic tree was reconstructed with 1000 bootstrap based on a Kimura 2-parameter model (Figure 3). The phylogenetic tree grouped the six Indonesian cattle in two major clusters: i.e. cluster *Bos javanicus,* which corresponds to Bali cattle, and cluster *Bos indicus* that corresponds to Kuantan cattle, Pesisir cattle, and PO. Intriguingly, two breeds (Madura cattle and Pasundan cattle) existed at a mixed group of *Bos javanicus* and *Bos indicus*. The result of this study did not show the closeness between Bali cattle and Bos taurus.

4. Discussion

This study reports, for the first time, the genetic diversity and phylogenetic analyses among Indonesian native

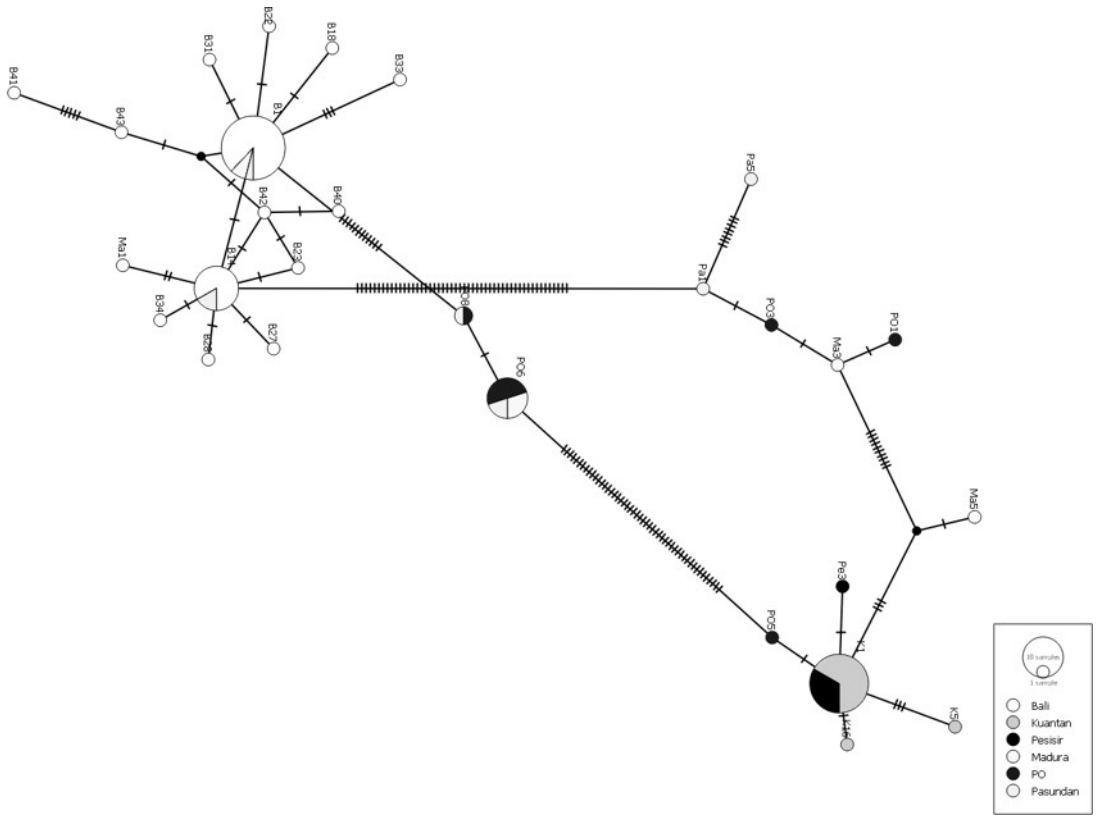

Figure 2. Median-joining network of haplotypes based on 16srRNA complete sequences.

Table 5. Genetic distance value in six breeds Indonesian cattle based on 16S rRNA gene.

	Bali	Kuantan	Pesisir	Madura	PO	Pasundan
Bali	-					
Kuantan	0.973	-				
Pesisir	0.974	0.000	-			
Madura	0.176	0.665	0.667	-		
PO	0.273	0.542	0.544	0.106	-	
Pasundan	0.130	0.676	0.678	0.119	0.009	-

cattle (Bali) and local Indonesian cattle (Kuantan, Pesisir, Madura, Pasundan, and PO breeds) based on a complete 16S rRNA sequence. Some previous studies have identified the genetic diversity of Bali cattle using D-loop [5], cyt b [9], and COI [20] markers, but the number of samples appears limited. Our study also identified genetic diversity in Kuantan and Pasundan cattle. Based on our investigation, the genetic diversity and phylogenetics of these cattle based on mtDNA markers are still limited.

16SrRNA mtDNA gene is a suitable marker for genetic diversity and phylogenetic studies because it contains a high polymorphism [10]. The analysis of genetic diversity offers an essential role in the genetic improvement and selection of cattle breeds for sustainable breeding and management programs of countries. The number of haplotypes constitutes one of the maternal line's key indicators in genetic diversity. The detected 14 haplotypes in Bali cattle indicated higher genetic diversity of 16S rRNA sequences. The number of haplotypes based on 16S rRNA sequence was higher than cyt b mtDNA sequence in the Bali, Madura, and Pasundan cattle [8,9]. Otherwise, the number of haplotypes in PO cattle was lower than the partial cyt b sequence [7].

In this work, haplotype diversity of Bali cattle was higher than other Indonesian local cattle breeds such as Pasundan, Madura, PO, Pesisir, and Kuantan cattle. We

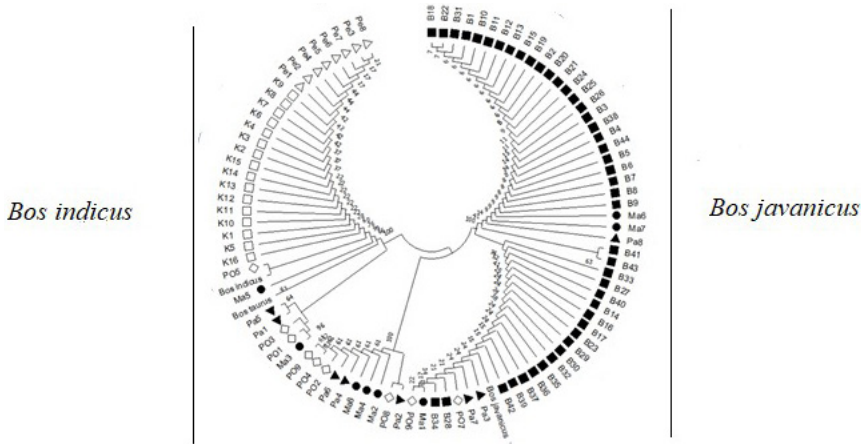

Figure 3. Phylogenetic tree of Bali and Indonesia local cattle based on 16srRNA complete sequence (B: Bali, Ma: Madura, K: Kuantan, Pe: Pesisir, Pa: Pasundan, PO: Peranakan Ongole; *Bos javanicus* GeneBank No. JN632606.1; *Bos indicus* GeneBank No. AY126697.1; *Bos taurus* GeneBank No. DQ 124418.1).

also noted that Bali, PO, Pasundan, and Madura cattle had higher genetic diversity with a haplotype diversity of Hd ≥ 0.70. Only Kuantan and Pesisir cattle had a lower genetic diversity, with an estimated haplotype diversity of Hd ≤ 0.25.

Haplotype diversity of Bali cattle (0.70 ± 0.063) in 16S rRNA sequence, higher than values in mtDNA D-loop sequence (0.614 ± 0.130) [5], but lower than values in the cytb sequence [9]. Haplotype diversity of Madura cattle (0.86 ± 0.108) in 16S rRNA is also lower than the value in mtDNA cytb sequence [9]. However, haplotype diversity of 16S rRNA sequence is higher than cytb sequences in Pasundan cattle [8]. We cannot compare haplotype diversity based on 16S rRNA in Pesisir and Kuantan cattle with other markers. Information about genetic diversity in three local cattle breeds is still limited.

The genetic diversity estimates based on nucleotide diversity (π) and haplotype diversity (Hd) parameters have been reported in Chinese native cattle using complete 16S rRNA sequence data [10]. Compared to their study, our analyses showed that the genetic diversity of Bali cattle was much lower than that found in Chinese native cattle.

Additionally, this present work demonstrated two major groups that successfully distinguished Bali and other Indonesian local cattle clusters from each other, i.e. cluster *Bos javanicus* and cluster *Bos indicus*. Bali cattle were separated from Kuantan and Pesisir cattle. In general, the results of the phylogenetic analysis were consistent with former studies. A previous study reported the origin of Bali cattle as Banteng (*Bos javanicus)* based on D-loop sequence [5], cyt b [9, 21], cytochrome oxidase subunit 1 (COI) [20]. Using microsatellite markers, Bali cattle were classified within the Bos javanicus cluster [22]. In terms of genetic distance, Bali cattle were separated clearly from *Bos indicus*, especially Pesisir and Kuantan, but they were closely related to Madura and Pasundan cattle.

Based on phylogenetic studies, we also identified Madura cattle in two separate clusters: Bos javanicus and Bos indicus. As explained in a previous study, Madura cattle is the crossbreed between Bali cattle (*Bos javanicus*) and Zebu cattle (*Bos indicus*) from India [1]. Based on maternal lineage using cyt b and D-loop mtDNA markers, Madura cattle can be grouped into two types, type I originating from *Bos indicus* and type II originating from *Bos javanicus* [23]. The crossing between *Bos javanicus* and *Bos indicus* was then more intensively carried out at the time of the government's promoting the development of Ongole cattle (*Bos indicus*) in the days of the Dutch East Indies.

Pasundan cattle are one of the local Indonesian cattle massively found as a natural population in West Java. These cattle are crossbred between *Bos indicus* and *Bos javanicus*. This study observed that Pasundan cattle have a close genetic relationship with Madura cattle and Bali cattle. This result corresponds well with the previous study, which used cytochrome oxidase subunit 1 (COI) [24] and microsatellite markers [25].

Another local Indonesian cattle, the Pesisir cattle breed, was separated from Bali cattle and existed in one cluster with *Bos indicus* cattle. Pesisir cattle were genetically close to Indian zebu cattle [2]. A microsatellite marker showed Pesisir cattle were genetically close to *Bos indicus* cattle and to be different from Bali cattle (*Bos javanicus*) [25]. Some studies reported that the Indonesian government imported Ongole zebu, and it was incorporated in breeding studies on Java and other Indonesian islands with increasing contributions, starting by the end of the 19th century [23].

According to the Minister of Agriculture, Indonesia, Kuantan cattle were classified as the newest Indonesian

local cattle. Our work demonstrated that based on mtDNA 16S rRNA, they were genetically close to Pesisir cattle. Following phenotypic analysis, Kuantan cattle is also found to be closely related to Pesisir cattle. This result also indicated these cattle have the same group as *Bos indicus*. A previous study showed some Indonesian local cattle have the closest relationship with *Bos indicus* and have been influenced by *Bos taurus* [6].

In conclusion, Indonesian native cattle have a high genetic variability in 16SrRNA mtDNA region. Phylogenetic analysis indicated the Bali cattle as *Bos javanicus* cluster.

This study showed a mixture of origin of local Indonesian cattle, between *Bos javanicus, Bos indicus,* and *Bos taurus*. Thus, the mtDNA 16S rRNA was a suitable marker for phylogenetic relationship analyses of Indonesian native and local cattle breeds.

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