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Haplotype and phylogenetic diversity using mitochondrial 12S rRNA gene marker in Bali cattle

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ABSTRACT

Bali cattle is one of Indonesia's original livestock genetic resources. The 12S rRNA can be used as a marker of genetic diversity, and until now, there has been no report on Bali cattle. The study was aimed to analyze the gene of mtDNA 12S rRNA to determine haplotype diversity and phylogenetics in Bali cattle populations of the eastern region of Indonesia. A total of 95 blood samples used consisted of three different populations which were Bali cattle, Ongole crossbred (PO) and Madura cattle as a comparison. This research was analyzed using PCR and sequencing methods. The data were analyzed using the cluster W method for estimating genetic distances, calculating diversity, and reconstructing phylogenetic trees using the MEGAX, DNAsP and Network. The genetic distance values ranged from 0.00200 to 0.01508, and the Haplotype diversity values ranged from 0.66000 to 0.91111. The nucleotide diversity values ranged from 0.00174 to 0.01673. There were 16 haplotypes found. The values of Gst, Nst and Fst were 0.00803, 0.07550, 0.07622; respectively. Based on the analysis, there were differences between Bali cattle from various populations, there were specific haplotypes. The 12S rRNA gene can be used as a genetic marker for diversity studies in Bali cattle and other cattle breeds although the diversity is low.

Keywords : Bali cattle, 12S rRNA gene, Genetic diversity, Phylogenetic tree

INTRODUCTION

The eastern region of Indonesia is one of the centers for cattle production in Indonesia with a large area of grazing lands. The beef cattle development pattern in the eastern region is generally carried out on infertile soil, water shortages, and high temperatures. One of the cattle breeds widely raised in eastern Indonesia is the Bali cattle (Purba and Hadi, 2016). Bali cattle distribution areas by province with a population of over 90,000 cows and a population growth of 2.8-5.9% include the provinces of South Sulawesi, Bali, East Nusa Tenggara, West Nusa Tenggara, South Sumatra, North Sulawesi, Gorontalo, South Kalimantan, Southeast Sulawesi, West Sulawesi and Lampung (Romjali, 2019). The distribution of Bali cattle, which is quite broad in various regions with different environmental conditions, makes the appearance of Bali cattle

between regions differ phenotypically. The Bali cattle diversity is thought to be due to cross-breeding with local cattle or the result of cattle adaptation to a different environment. Species phenotypic responses may occur due to specific environmental stimuli and are known as "phenotypic flexibility" (Xue *et al.,* 2019). The species spread over a large area and may cause genetic variation.

Bali cattle is one of the original Indonesian germplasm that needs to be developed and preserved. Bali cattle is designated as a native Indonesian cattle based on the Decree of the Minister of Agriculture Number 325/Kpts/ OT.140/1/2010 concerning the Determination of Bali Cattle Clusters. One of the advantages of Bali cattle is their excellent adaptability. Besides being a vital genetic resource, Bali cattle have become a world asset based on the FAO data (Hikmawaty *et al.,* 2014). Bali cattle is the result of the domestication of wild bulls. Various names are given to the bull, including the Burmese bull (*Bos javanicus birmanicus*), Javanese bull (*Bos javanicus javanicus*), and Bornean or Bornean bulls (*Bos javanicus lowii*) (Martojo, 2012). Based on Appendix 1 list of IUCN Red Data and U.S. Endangered by law, the bison species is classified in the endangered animal group (Rahman *et al*., 2019; Gardner, 2016). Intensive crossbreeding programs for Bali cattle through natural mating and artificial insemination using exotic breeds may cause extinction due to uncontrolled crossing. Introgression and crossbreeding that is not well planned will contribute the most to the loss of native breeds, thus raising concerns in the future regarding the existence of local cattle (Senczuk *et al*., 2020).

Understanding haplotype diversity and population structure, besides being used to understand the evolutionary history of national origins, is also valuable for providing important information for biodiversity conservation and management programs to maintain their existence and avoid extinction. MtDNA has become a popular research object for analyzing the genetic diversity of a population (Zhao *et al*., 2013). MtDNA is maternally inherited without recombination and has a high mutation rate. The vertebrate mitogenome organization and salient features have made mtDNA a popular marker in the last two decades. Eukaryotic cells contain mitochondria, with a few exceptions. The mitochondrial genome consists of a double-stranded DNA molecule with a length of about 16 kb, accounting for 1% - 2% of the total DNA in mammalian cells (Yang *et al.* 2014). Mitochondrial DNA encodes 37 genes (2 ribosomal RNA (rRNA) ((small (12S) and large (16S))), 22 parts of transfer RNA (tRNA), and 13 parts of the protein (polypeptides), all of which are the components of the mitochondrial oxidative phosphorylation system (OXPHOS)) (Shokolenko and Alexeyev, 2022). These ribosomal RNAs (12S rRNA) encoded by the mitochondrial genome are required to translate messenger RNA into mitochondrial proteins. The 12S rRNA genes occupy 1/16 of the entire mitochondrial genome, and these two mitochondrial genes have many nucleotide substitutions (Yang *et al*., 2014).

The 12S rRNA mtDNA gene can detect haplotype and phylogenetic diversity in livestock. Phylogenetic relationships can be identified through gene sequence analysis by identifying the genetic diversity, haplotypes, and genetic distances (Liu *et al.,* 2013). Mitochondrial DNA (mtDNA) polymorphisms have been widely used to determine phylogenetic relationships in cattle breeds. The results of this 12S rRNA sequence have been used to identify the type of milk in the cattle of sheep and goats (Tortorici *et al.,* 2016), to identify the types of livestock meat (Islam *et al.,* 2021), to identify the presence of a mixture of dog and rat meat in beef (Cahyadi *et al.,* 2019), and to detect pork and beef in food products (Roy *et al.,* 2021). Based on these reasons, the 12S rRNA gene can be used as a marker of genetic diversity, and until now, there has been no report on Bali cattle in particular. Thus this study aimed to analyze the gene of mtDNA 12S rRNA to determine haplotype diversity and phylogenetic in Bali cattle populations of the eastern region of Indonesia.

MATERIALS AND METHODS

The research was conducted from October 2022 to December 2022. The research was conducted at the Animal Molecular Genetics Laboratory, Department of Animal Production and Technology, Faculty of Animal Seience, Bogor Agricultural University. The samples used in this study were 95 blood samples taken from the collection of Animal Molecular Genetics Laboratory, Faculty of Animal Science, Bogor Agricultural University. The samples were taken by random sampling method. The criteria cattle were maturity of body and sex (Table 1).

DNA Extraction

DNA extraction was carried out using the DNA Mini Kit (gSYNC^{TM} Paint. No. GS100). In the first stage, a blood sample stored in 70% alcohol was taken as much as 400 µL in a microcentrifuge tube of 1.5mL. Next, 1000 µL of PBS solution was added to the microcentrifuge tube and was homogenized with vortex for 10 seconds. Then, it was centrifuged at 12000 rpm for 5 minutes, and the supernatant was discarded. In the next stage, 200 µL GST buffer and 20 µL Proteinase K were added and were homogenized using a vortex for 10 seconds, and then it was incubated at 60°C for 16 hours while shaking. The sample was then centrifuged at 16000 rcf for 2 minutes. Then, the supernatant was transferred into a new microcentrifuge tube of 1.5 mL, 200 µL GSB buffer was added, and homogenized with vortex for 10 seconds. Next, 200 µL absolute ethanol was added and homogenized with vortex for 10 seconds. Next, the GS column was prepared and was placed into a 2 mL collection tube. The samples added with 200 µLabsolute ethanol were transferred to the GS column and was centrifuged at 16000 rcf for 1 minute. Then, the supernatant in the collection tube was discarded.

The sample in the GS column was added with 400 µL W1 buffer and was centrifuged at 16000 rcf for 30 seconds. After the centrifugation process was complete, the supernatant was discarded. The next step was to add 600 µLwash buffer, which had been added with absolute ethanol and was centrifuged at 16000 rcf for 30 seconds. Then, the supernatant was discarded. Next, the samples were centrifuged again, drying at 16000 rcf, and was carried out for 3 minutes. Next, the GS column was transferred to the fresh and clean microcentrifuge tube of 1.5 mL. Next, 100 µL elution buffer was added, preheated, and allowed to stand for 3 minutes. Then, the samples were centrifuged for 30 seconds to obtain the extracted DNA.

12S rRNA mtDNA Sequence Amplification and Sequencing

The primers used to amplify the complete 12S rRNA sequence consist of two pairs of primers. Primer preparation was carried out using

Table 1. Breed, sex, number of animal, and sampling location of cattle used in the study

Cattle Breed	Sex	Number of animals	Location	Time of blood sample collection
Bali	Male	25	Breeding Centre Denpasar, Bali	2014
Bali	Female	25	Breeding Centre Serading, West Nusa Tenggara	2013
Bali	Female	25	*village breeding centr*e (VBC), Baru District, South Sulawesi	2013
Madura	Female	10	*village breeding centr*e (VBC), Pamekasan District, East Java	2013
PO*	Female	10	*village breeding centr*e (VBC), Kebumen District, Central Java	2019
Total		95		

*PO: Ongole crossbred

the 12S sRNA sequence from GenBank for Nellore cattle (AY126697.1).

The primer was designed using primer3 software (http://primer3.ut.ee), and the primer potency was evaluated using PCR Primer Stats (PCR Primer Stats (northwestern.edu). The sequence of the primary sequences is presented in Table 2.

In addition, the Gaur sequence (*Bos gaurus*), Gayal (*Bos frontalis*), *Bos indicus,* and *Bos taurus* were used as the comparison. The Genbank access numbers for the comparison sequences are presented in Table 3.

The total PCR reaction volume used was 25 µl, consisting of 2 µl DNA sample (50-100 ng), 0.3 µl each forward and reverse primer (25 pmol/ µL), 12.5 µl red mix, and 9, 9 µl distilled water. Amplification was performed using a thermocycler (GeneAmp® PCR System 9700, Applied Bio SystemTM, Foster City, CA, USA) under the following conditions: pre-denaturation at 95 °C for 1 minute, 35 cycles of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 15 seconds, extension at 72 °C for 10 seconds, and final extension at 72 °C for 5 minutes.

Electrophoresis

PCR products were visualized using gel electrophoresis of 1.5%. Electrophoresis of PCR products was carried out using 3 µL of PCR products on 1.5% agarose gel for 30 minutes. The steps in making agarose gel were to mix 0.45 g agarose powder and 0.5 x TBE 30 mL and then heat in the *microwave* (± 3 minutes). In the following process, the agarose solution was cooled using *a magnetic stirrer* at 50 rpm (± 2 minutes). Next, agarose solution was added with *PeqGreen* of 1 µL, was homogenized (± 2 minutes), was placed into the mold, and left for 30 minutes at room temperature.

The prepared agarose gel was put into the electrophoresis bath filled with *buffer* (0.5 x TBE). Then, 3 µL amplicon was put into the well and *marker* 100 bp at 100 V (± 30 minutes). Agarose gel after electrophoresis was carried out to determine the length of the DNA bands using UV *Transilluminator*.

Sequencing

Forward and reverse of 12S rRNA gene PCR products were sequenced using a company sequencing service at 1st Base, Selangor, Malaysia.

Data Analysis

The sequencing results were carried out to validate the data using the Bio Edit and FinchTV programs. Data analysis of complete 12S rRNA mtDNA gene sequences was aligned using the cluster W method, and genetic distances were calculated using p distance in the MEGA program version 10. Nucleotide diversity expressed

Table 2. Primer sequence used for 12S rRNA gene amplification

Gene	Sequence (5'-3')	Product length (bp)	Annealing temperature (^{o}C)
12S rRNA	F-CAC ATA GGT TTG GTC CCA GC R-AGG TGT AAA CTG GAT GCT TTG T	995	60

Table 3. GenBank access number of the mitochondrial genome and 12S rRNA gene length

Species	GenBank ID	Complete sequence (bp)	12S rRNA (bp)
Gaur (*Bos gaurus*)	MT360653.1	16345	956
Gayal (*Bos frontalis*)	MW763078.1	16347	956
Bos indicus	AY126697.1	16341	956
Bos taurus	NC_006853.1	16338	956

as the number of polymorphic sites (S), nucleotide differences (K), haplotype diversity (Hd), and nucleotide diversity (π) was calculated using the DNAsp version 6.12.01 program. The median-joining network was analyzed using the Network program version 10.2. The phylogenetic tree was reconstructed using the bootstrapped maximum likelihood method with 1000 repetitions, and the gamma distribution (+G) used five rate categories.

RESULTS AND DISCUSSION

The complete 12S rRNA mtDNA gene sequence was successfully amplified at an annealing temperature of 60^0C with the sequence length used to analyze 909 bp. An example of the results of the amplification of the complete 12S rRNA sequence can be seen in Figure 1.

The genetic distance between Bali cattle from various regions and other local Indonesian cattle obtained by aligning the existing nucleo-

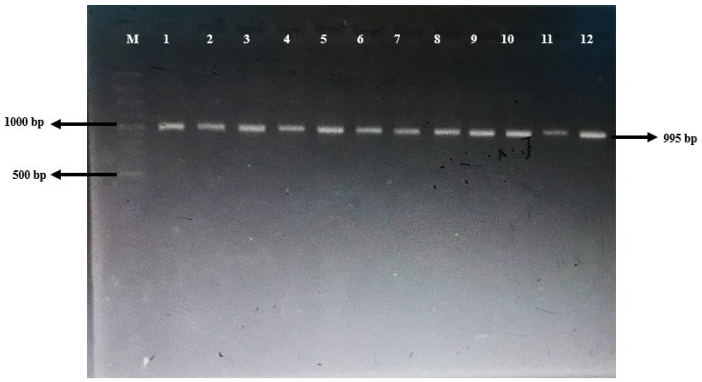

Figure 1. An example of the results of the amplification of the complete 12S rRNA gene **(**1-12 =Number of samples; M=100 bp Marker**)**

Table 4. Genetic distance between the Bali and other cattle breeds

Breed	1	2	3	4	5
PO [1]	-				
Madura [2]	0.01508	-			
Bali from Denpasar [3]	0.00875	0.01005	-		
Bali from South Sulawesi [4]	0.00941	0.01049	0.00201	-	
Bali from West Nusa Tenggara [5]	0.00944	0.01048	0.00200	0.00211	-

Table 5. Diversity of 12S rRNA mtDNA sequences in Bali cattle from various regions and other local cattle

Breed	N	S	H	K	Hd	π
Bali from Denpasar	25	5	5	1.580	0.660	0.00174
Bali from South Sulawesi	25	5	5	1.993	0.686	0.00220
Bali from West Nusa Tenggara	25	5	5	1.953	0.750	0.00216
Madura	10	43	7	15.155	0.911	0.01673
PO	10	51	5	12.822	0.666	0.01415
Overall	95	57	16	4.721	0.722	0.00521

N = Number of sample; S= Segregating Site; H= Number of haplotype; K= nucleotide Differences; Hd= Haplotype Diversities; π= Nucleotide Diversity

Table 5. Diversity of 12S rRNA mtDNA sequences in Bali cattle from various regions and other local cattle

Breed	N	S	H	K	Hd	π
Bali from Denpasar	25	5	5	1.580	0.660	0.00174
Bali from South Sulawesi	25	5	5	1.993	0.686	0.00220
Bali from West Nusa Tenggara	25	5	5	1.953	0.750	0.00216
Madura	10	43	7	15.155	0.911	0.01673
PO	10	51	5	12.822	0.666	0.01415
Overall	95	57	16	4.721	0.722	0.00521

N = Number of sample; S= Segregating Site; H= Number of haplotype; K= nucleotide Differences; Hd= Haplotype Diversities; π= Nucleotide Diversity

Table 6. Genetic Differentiation between Populations

Genetic differentiation	Value	Gene flow (Nm) (tail/generation)
Gst	0.00803	30.88
Nst	0.07550	3.060
Fst	0.07622	3.030

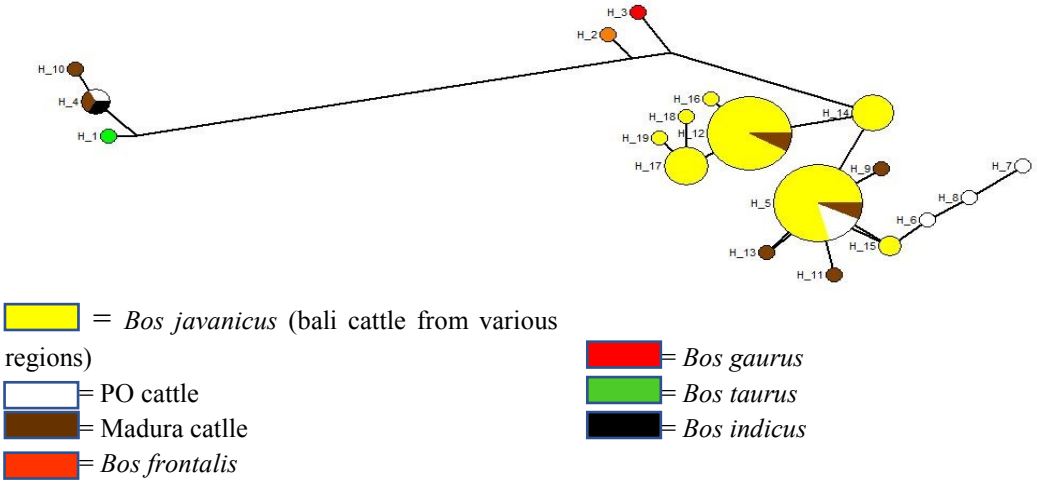

Figure 2. Analysis results for haplotype networks in Bali cattle from various regions

tides can describe the inter-species kinship. Genetic distance values in this study ranged from 0.00200 to 0.01508 (Table 4). The lowest genetic distance was found between Bali cattle from Denpasar and Bali cattle from West Nusa Tenggara (0.00200). In contrast, the most significant genetic distance was found between PO and Madura cattle. Bali cattle between regions show genetic distance values ranging from 0.00200 to 0.00211. Low genetic distance results in low genetic variation, contributing to livestock phenotype and adaptation (Takeshima *et al*., 2018). Genetic variation can be associated with geographic isolation in populations, selection, mutation, and adaptation to different ecologies (Madilindi *et al*., 2019).

Genetic diversity is essential information in breeding and conservation programs (Misrianti *et al*., 2022). The sequencing results showed that the 12S rRNA gene sequences of Bali cattle in

Table 7. Haplotype distribution among Bali and local Indonesian cattle

Haplotype	Breed								
	Bos taurus	*Bos frontalis*	*Bos gaurus*	*Bos indicus*	PO	Madura	Bali from Denpasar	Bali from South Sulawesi	Bali from West Nusa Tenggara
H1	1								
H2		1							
H3			1						
H4				1	1	1			
H5					6	3	13	11	10
H6					1				
H7					1				
H8					1				
H9						1			
H10						1			
H11						1			
H12						2	7	9	7
H13						1			
H14							3		3
H15							1	1	
H16							1		
H17								3	4
H18								1	
H19									1

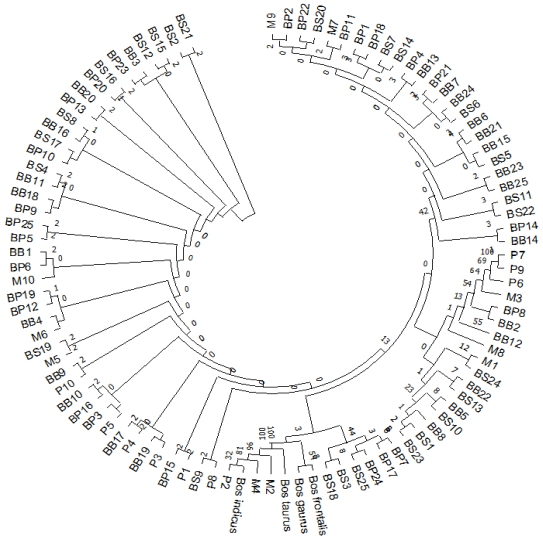

Figure 3. Phylogenetic tree in Bali cattle from various regions

various regions and other local Indonesian cattle were polymorphic. The total polymorphic site found in the study were 57 site, the highest number of polymorphic site was found in PO cattle (51 site), while the number of polymorphic site for Bali cattle in various regions had the same number (5 site). The mutation position and genotype of 12S rRNA gene in Bali cattle from various region can be clearly seen in Figure 4 and Figure 5. The total haplotypes found in the study were 16 haplotypes. The highest number of haplotypes was found in Madura cattle, while the number of haplotypes for Bali cattle in various regions and PO cattle had the same number.

Figure 4. Sequence of 12S rRNA gene in Bali cattle from various regions.

Haplotype diversity ranged from 0.6600 to 0.91111. Nucleotide diversity values ranged from 0.0017 to 0.01673 (Table 5). The haplotype diversity of Bali cattle from Denpasar was lower than that of Bali cattle from other regions and other Indonesian local cattle where haplotype diversity was the highest among the population of cattle studied; Madura cattle (0.91111). According to Yan *et al*. (2019), there is different diversity among different mtDNA genes in cattle. So, if livestock has high haplotype diversity, the diversity value is abundant in the cattle breed.

The genetic differentiation between populations used three measures (Table 6). The Nm value reflects the number of individuals that migrate between populations per generation (Feder *et al*., 2019). The analysis results showed that the Gst value between populations was 0.00803, with the gene flow value of Nm = 30.88. The value of Nm > 1 indicated the level of gene flow between populations able to overcome genetic drift. According to Alcala *et al.* (2014), the Gst value measures genetic differences between populations based on allele fixation. Variation in Gst values is from 0 to 1. The genetic differences between the five local Indonesian cattle populations were classified as low based on the criteria for variations in Gst values. This low value showedthat the total genetic diversity came from genetic diversity between populations of 0.80%, while 99.2% came from genetic diversity within the population. The Nst value in the study was 0.07550, which can be used to estimate population subdivision at the nucleotide level and fully parallels the Fst value. Zainudin and Naim (2018), Examination of gene flow at the nucleotide level can use the Nst value derived from DnaSp.

The Fst value can provide essential insights into the evolutionary processes that influence the structure of genetic variation within and between populations, as well as one of the most widely used descriptive statistics in population and evolutionary genetics. The Fst value in the study was 0.07622. Soheila *et al*. (2021) stated, Fst values between 0-0.05 indicate a low category of genetic differentiation in the population. The Fst value of 0 indicates that the population shares genetic material through high-level breeding. Fst values for mammals generally range from 0 to 0.25, with most values approaching 0.1. Fst is directly related to the variance of allele frequencies between populations and vice versa with the level of similarity between individuals in the population. If the first is small, the allele frequencies in each population are similar. If the first is large, the allele frequencies are different. Sihite *et al.* (2019) stated that the population of Bali cattle in BPTU-HPT Denpasar has undergone selection, so no located in Hardy-Weinberg equilibrium. A population was in Hardy-Weinberg equilibrium if there was no selection, mutation, migration and genetic drift.

According to the classification under the genus *Bos*, cattle are divided into several species, including *Bos gaurus*, *Bos javanicus*, *Bos indicus*, and *Bos taurus* (Syed-Shabthar *et al*., 2013). Meanwhile, *Bos frontalis* or gayal is often considered the domestic form of gaur. However, Gaur and Gayal differences may have increased with recent Zebu introgression into the Gayal from the Yunnan population, which is known to contain zebu mtDNA (Indian cattle) (Zhang *et al*., 2020). Therefore, in this study, the sequencing data must be compared with GenBank data for several cattle breeds.

The results of haplotype distribution analysis from 95 local Indonesian cattle sequences and four sequences from the GenBank data show that each local cattle population in Indonesia had a unique haplotype (Table 7 and Figure 1). The PO cattle studied had the same haplotype as *Bos indicus*, which was H4, and there were also populations of Madura cattle with the same haplotype. All Indonesian local cattle populations share the haplotype with most individuals in H5. Bali and Madura cattle from various regions have the same haplotype in H12. This research found specific haplotypes between Bali cattle from different regions and other Indonesian local cattle breeds, which have not been exposed so far. The specific haplotypes of H6, H7, and H8 were only found in PO cattle, and H9, H10, H11,

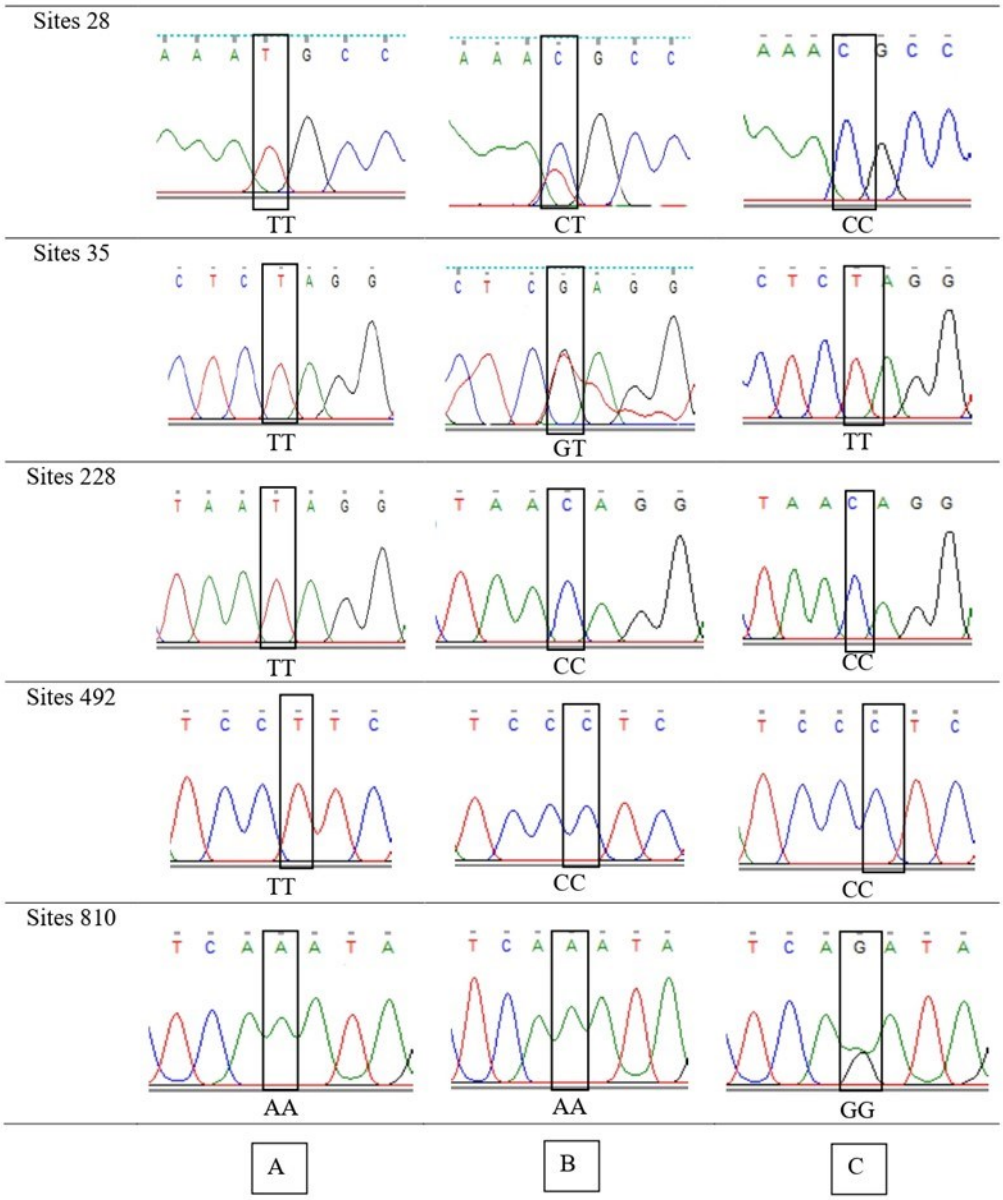

Figure 5. Chromatogram of mutation position and genotypes of 12S rRNA gene in Bali cattle. (A) Bali cattle from Denpasar, (B) Bali cattle from South Sulawesi, (C) Bali cattle from West Nusa Tenggara.

and H13 were found in Madura cattle. This study revealed the specific haplotype markers for Bali cattle for each region, i.e., H16 for Bali cattle from Denpasar, H18 for Bali cattle from South Sulawesi, and H19 for Bali cattle from West Nusa Tenggara. According to Leitwein *et al*. (2020), haplotype information can assess historical demography, gene flow, selection, and evolutionary outcomes of hybridization across different timescales relevant to conservation concerns. Haplotype data also contribute to understanding genetic mix by characterizing local genomic ancestry, which allows dissection of variation in introgression rates across genomes, thereby shedding light on evolutionary processes in ancestry.

Median-joining network haplotypes were reconstructed using the Network program (Figure 2). The existence of specific haplotypes or unique haplotype markers in local Indonesian

cattle, both PO cattle, Madura cattle, and Bali cattle in various regions indicates a need for reasonable strategic steps going forward for local Indonesian cattle breeding programs so that the original Indonesian germplasm is maintained. Systematic relationships in livestock will enable maps of the history, origin, and distribution of cattle breeds, which can assist in developing more rational breeding and conservation programs (Shabthar *et al*., 2013). Knowledge of the genetic diversity and structure of native livestock populations is invaluable to the livestock industry, especially in tropical developing countries. Genetic diversity is the basis for improving genetics through well-designed and practical breeding (Zwane *et al*., 2019).

Grouping analysis of 95 individuals in five local Indonesian cattle populations compared to four sequences of cattle breeds from the Genbank produced a phenogram, as shown in Figure 3. Bali cattle from various regions were differences, there were specific haplotypes between Bali cattle in various regions. However, some mixed blood from the Indonedia local cattle breed, such as PO and Madura causes dcreasing the level of purity, and vice versa. The variation may be due to the distribution of cattle, which also adapt to the rearing environment. For example, the government intensively crossed *Bos javanicus* and *Bos indicus during* the Ongole cattle development program (*Bos indicus*) during the Dutch East Indies era (Misrianti *et al*., 2022). This introgression event is like what happened to Zebu cattle (*Bos indicus*) in South China which contains 3% of the Banteng component (*Bos javanicus*) and causes the cattle to have genes related to sensory perception, immunity, heat acclimatization, and color patterns (Chen *et al*., 2018). Then, vice versa, the introgression of Indian cattle to *Bos javanicus* resulted in the presence of genes that regulate behavior in livestock (Zhang *et al*., 2020).

CONCLUSION

Bali cattle have specific haplotypes that distinguish three locations, such as haplotypes H16 (from Denpasar), H18 (from South Sulawesi), and H19 (from West Nusa Tenggara). Genetic distance and phylogenetic analysis showed that Bali cattle are different in various regions. This study showed that the 12S rRNA gene can be used as a genetic marker to determine diversity in beef cattle, especially in Bali cattle, although diversity is low.

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