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Diversity of D-loop mitochondrial DNA (mtDNA) sequence in Bali and Sumba Ongole cattle breeds

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ABSTRAK

Penelitian ini bertujuan untuk mendapatkan keragaman sekuen lengkap DNA mitokondria D-loop (mtDNA) pada sapi Bali dan Sumba Ongole (SO). Sebanyak 24 sampel darah diperoleh dari sapi Bali (19 ekor) dan sapi SO (5 ekor), diekstraksi dan kemudian dianalisis untuk mendapatkan sekuen mtDNA D-loop. Pensejajaran sekuen mtDNA D-loop dilakukan menggunakan metode *clustal W*. Jarak genetik dihitung menggunakan metode *p*-distance, sedangkan pohon genetik dibangun menggunakan metode *Neighbor-Joining* (NJ) dengan menggunakan program MEGA 6. Jumlah haplotipe, keragaman haplotipe (Hd) dan keragaman nukleotida (Pi) dianalisis menggunakan program DnaSP versi 6. Diperoleh sekuen mtDNA D-loop pada sapi Bali yaitu panjang 921-1119 bp dan sapi SO 913 bp dan masing-masing memiliki 8 dan 4 haplotipe. Nilai Hd dan Pi sapi Bali masing-masing 0,625 ± 0,139 dan 0,0266 ± 0,0145, sedangkan sapi SO yaitu 0,900 ± 0,1610 dan 0,0064 ± 0,0015. Ditemukan 22 bp nukleotida berulang pada sapi Bali dengan jumlah ulangan 3-9 kali dengan panjang 66-198 bp di fragmen mtDNA D-loop. Sekuens berulang tersebut tidak ada pada ternak SO. Jarak genetik dan pohon genetik di daerah *hypervariability* (HV-1) mtDNA D-loop (166 bp) menghasilkan kluster yang jelas berbeda antara *Bos javanicus*, *Bos indicus*, dan *Bos taurus*.

Kata kunci: sapi Bali, D-loop MtDNA, keragaman genetik

ABSTRACT

This study aimed to investigate the diversity of the complete sequence of D-loop mitochondrial DNA (mtDNA) in Bali and Sumba Ongole (SO) cattle breeds. A total of 24 blood samples were collected from Bali cattle (19 heads) and SO cattle (5 heads), and were extracted and then analyzed to obtain the sequence of D-loop mt DNA. Multiple alignments of the whole sequence of D-loop mtDNA were determined using clustal W. Genetic distance was calculated using a p-distance method, while the genetic tree was constructed using neighbor-joining (NJ) based on MEGA 6. Haplotype number, haplotype diversity (Hd) and nucleotide diversity (Pi) were analyzed using DnaSP version 6. As a result, the sequence of D-loop mtDNA in Bali cattle (921-1119 bp) and SO cattle (913 bp) was reported to have 8 and 4 haplotypes. Hd and Pi of Bali cattle reached 0.625±0.139 and 0.0266±0.0145, respectively, which wwere different from that of SO cattle, namely 0.900±0.1610 and 0.0064±0.0015, respectively. Specifically, we found 22 bp-repetitive nucleotide in Bali cattle, existing 3-9 times with a length of 66-198 bp present in D-loop mtDNA. This unique feature did not exist in SO cattle. Genetic distance and genetic tree determined according to sequence in hypervariability (HV-1) region of D-loop mtDNA (166

bp) resulted in satisfied separation, successfully classifying *Bos javanicus*, *Bos indicus*, and *Bos taurus* cluster.

Keywords: Bali cattle, D-loop MtDNA, genetic diversity

INTRODUCTION

The most popular and important livestock genetic resources for small holder farmers in Indonesia are Bali cattle (*Bos javanicus*) and Sumba Ongole (SO) cattle (*Bos indicus*) (Mohamad *et al*., 2009). Ongolization program, which is a hybridization of Bali cattle with SO cattle breeds, produces Ongole Grade or Peranakan Ongole (PO) cattle and other breeds in Indonesia such as Madura, Pesisir and Aceh cattle breeds (Martojo, 2012; Hartati *et al.*, 2015). In 1970, the crossbreeding program was intensively conducted via artificial insemination (AI) (Purwantara *et al.*, 2012) and successfully producing SimPO and LimPO cattle (Trifena *et al.*, 2016). In short, the production of beef cattle in Indonesia is generally based on hybridization between Bali cattle and SO cattle (Nijman *et al.*, 2003; Lenstra *et al.*, 2014; Hartati *et al.*, 2015) and indeed, Bali cattle (*Bos javanicus*) genetically contributed to Southern Chinese cattle and was found at a very different cluster over other cattle breeds in the world (Gao *et al.*, 2017).

The historical and important role of Bali cattle and SO cattle towards the existence of current breeds in Indonesia evidenced their high adaptability to tropical condition. However, the genetic information of Bali cattle (*Bos javanicus*) has remained deficient in comparison with that of other breeds such as *Bos taurus* and *Bos inducus* (Sharma *et al.*, 2015; Doğan *et al.*, 2017). Regarding studies on genetic diversity and phylogenetic, mitochondrial DNA genome could be the best approach (Rehman *et al.*, 2017) compared to markers from the nuclear genome (Van Marle-Köster and Nel, 2003; Viryanski, 2019). Mitochondrial genome was reported to have a high variability, which is maternally inherited with no recombination (Lenstra *et al.*, 2014), in which the DNA occurred in the form of circular and supercoil molecules with length of 16kb in livestock (Rehman *et al.*, 2017); meanwhile, in *Bos taurus* and *Bos indicus*, it was found to reach 16.338 and 16.339 nucleotides (Hiendleder *et al.*, 2008). A specific area responsible for controlling genes (37 genes) present in the mitochondrial genome was D-loop comprised of 922 nucleotides (Lee *et al.*, 2012). For this reason, D-loop mitochondrial is often employed to analyze genetic tree and genetic diversity of cattle breeds (Yang *et al.*, 2014; Sharma *et al.*, 2015; Villegas Castagnasso *et al.*, 2015; Srirattana *et al.*, 2017; Pramod *et al.*, 2018; Xia *et al.*, 2019).

Currently, D-loop mitochondrial based on partial sequencing for investigation of genetic diversity was reported in some cattle breeds such as Aceh cattle (Sari *et al*., 2016), Bali cattle and Madura cattle (Nijman *et al.,* 2003). Besides, the D-loop mitochondrial analysis was also carried out in Bali cattle, Madura cattle, PO cattle, and Pesisir Sumatra Barat cattle (Sutarno, 2010) as well as SO cattle (Agung and Hermansyah, 2018) using polymerase chain reaction-fragment length polymorphism (PCR-RFLP). Furthermore, D-loop mitochondrial was also sequenced in Bali cattle, Madura cattle, Pesisir cattle, and Aceh cattle (Abdullah *et al*., 2012) despite using a limited number of samples. Based on our investigation, scientific evidence related to the complete sequence of D-loop mitochondrial in Bali cattle and SO cattle were rather scarce, which make our recent work highly important to conduct. Therefore, this present work aims at discovering the diversity of the D-loop mtDNA sequence in Bali cattle and SO cattle, contributing to future breeding programs for Bali cattle and SO cattle.

MATERIALS AND METHODS

Animal and Samples

The D-loop mitochondrial DNA (mtDNA) in this experiment was sourced from 19 individuals of Bali cattle (Breeding Center of Bali cattle in Bali island, Indonesia) and 5 individuals of SO cattle (from smallholder farmer in Nusa Tenggara Timur, Indonesia). Furthermore, we also employed a D-loop sequence from GenBank for cattle breeds including *Bos indicus* namely Nellore (AY126697.1), Ongole (AY378135.1) and Sahiwal (L27732.1), *Bos taurus* namely Simmental (AF034442.1), Limousin (AF034446.1) and Angus (AY676858.1).

Mitochondrial Genome Isolation, Amplification and Sequencing

The mitochondrial genome was isolated

from blood using the genomic DNA Mini Kit (GenAid protocol Cat. No. GB100). The fragment of D-loop mtDNA in Bali cattle and SO cattle was amplified using forward primer 5′-TAG TGC TAA TAC CAA CGG CC-3′ and reverse primer 5′-AGG CAT TTT CAG TGC CTT GC-3′ (Hassan *et al.* 2009) with modification by substituting two nucleotides in forward primer, i.e. 5'-TAG TAC TAA TAC CAA CAG CC-3' according to GenBank Accession No. AY126697.1 in which forward and reverse primer occurred at Cytb and tRNAPhe, which resulted in a sequence length of 1144 bp. PCR reaction volume was made up to 25 µL, consisting of 2 µL DNA sample (50-100 ng), 0.3 µL of each forward primer and reverse primer (25 pmol/µL), buffer (0.2 µL dNTPs, 1 µL $MgCl_2$, and 2.5 µL 10x Buffer), 0.1 µL (0.5 U) Taq Polymerase, and 18.9 µL pure water (DW). PCR equipment (Thermoscycler Applied Biosystems) was set as follows: initial denaturation 95°C (5 min), denaturation 95°C (30 sec, 35 cycles), annealing 60°C (45 sec), extension 72°C (60 sec) and final extension 72°C (5 min). PCR product was then electrophoretically analyzed using agarose gel 1.5% (0.5 g dissolved in 33 ml 0.5xTBE) and visualized using UV transilluminator. To collect D-loop mitochondrial sequence, PCR fragments were sequenced using ABI PRISM® 3730xl Genetic Analyzer BigDye® Terminator v3.1 (Applied Biosystems) with 8-capillary system provided by 1st BASE, Selangor Malaysia.

Sequencing Analysis

The sequence of D-loop mtDNA fragment was analyzed using multiple alignments according to the clustal-W method, while genetic distance was measured using p-distance. Besides, the genetic tree was analyzed using neighbor-joining (NJ) with bootstrap 1000 replication based on Molecular Evolutionary Genetics Analysis (MEGA 6) software (Tamura *et al.,* 2013). Genetic distance and genetic tree for Bali cattle and SO cattle was estimated from hypervariable region (HV-1) with sequence length of D-loop mtDNA reaching up to 166 bp, as well as for other cattle breeds based on sequence data from GenBank with accessed number of AY126697.1, Y378135.1, L27732.1, AF034442.1, AF034446.1 and AY676858.1 (http://www.ncbi.nlm.nih.gov). Haplotype variability and diversity of nucleotide in the D-loop mtDNA sequence of Bali cattle and SO cattle were determined by DnaSP version 6 (Rozas *et al*., 2017).

RESULTS

Diversity of D-loop mtDNA

The results showed that D-loop mtDNA in Bali cattle and SO cattle was in accordance with target fragment (Figure 1) with sequence length of 921-1119 bp and 913 bp, while variability of haplotype (Hd) and nucleotide (Pi) for Bali cattle and SO cattle was 0.625±0.139, 0.900±0.161 and 0.0266±0.0145, 0.0064±0.0015 (Table 1). Even though Bali cattle possessed a greater number of haplotype (8 haplotypes) than SO cattle (4 haplotypes), the variability of haplotype was higher in PO cattle than in Bali cattle. Also, the average number of nucleotide differences (k) and the number of the polymorphic site (s) were higher in Bali cattle than in SO cattle. We also found that the composition of nucleotide in D-

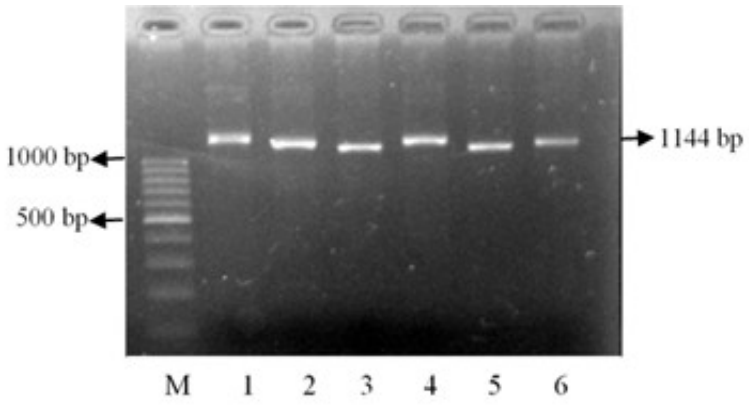

Figure 1. Gel Electrophoresis of PCR Product from a D-loop Mitochondrial Fragment in Bali Cattle SO Cattle. Lane M: marker 100 bp; lane 1-6: samples; bp means base pair

Table 1. Variability of D-loop MtDNA Sequence in Bali and Sumba Ongole Cattle

Breed	Size of D-loop (bp)	Haplotype Number	Average Number of Nucleotide Differences (k)	Number of Polymorphic Site (s)	Haplotype Diversity (Hd)	Nucleotide Diversity (Pi)
Bali	921	8	16.54	118	0.614±0.130	0.0250±0.0140
SO	913	4	5.80	12	0.900±0.161	0.0064±0.0015

Table 2. Frequency of Haplotype of D-loop mtDNA in Bali and Sumba Ongole Cattle

Breed	Haplotype Number	Frequency							
		Hap-1	Hap-2	Hap-3	Hap-4	Hap-5	Hap-6	Hap-7	Hap-8
Bali	8	0.05	0.63	0.05	0.05	0.05	0.05	0.05	0.05
SO	4	0.20	0.20	0.40	0.20				

loop mtDNA between A-T and G-C reached 60.4% and 39.6% for Bali cattle (921 bp), and 61.1% and 38.9% for SO cattle (913 bp). This suggested that no significant difference in nucleotide composition was found between Bali cattle and SO cattle, but A-T was higher than G-C. Haplotype 2 (Hap-2) in Bali cattle was observed as the highest frequency, while the highest one in SO cattle was haplotype 3 (Hap-3) (Table 2).

Interestingly, repetitive nucleotide was observed in the D-loop mtDNA of Bali cattle, but it did not exist in SO cattle (Figure 2). The presence of 22 bp repetitive nucleotide in Bali cattle is responsible for diversity within the population and dissimilar sequence length (Figure 2). Such repetitive nucleotide was 5'-GTA CAT AAT ATT AAT GTA ATA A-3' with repetition of 3-9 times and found as polymorphic within population; thus, we identified 5 types of repetitive nucleotide according to its replication, and in this case, the highest frequency was attributed to repetitive nucleotide type 1 (Table 3). Based on sequence reference of GenBank (AY126697.1), the repetitive nucleotide began with nucleotide with size of 15961-16159 (198 bp), which was specifically found in Bali cattle, while the position of nucleotide with size of 15795-15960 (166 bp) constituted a hypervariability (HV-1) and that with size of 16160-16341, 1-366 (755 bp) was detected as hypervariability (HV-2) (Figure 2).

Genetic Distance and Genetic Tree

The genetic distance and genetic tree among cattle breeds including *Bos taurus* and *Bos indicus*, Bali cattle, and SO cattle were formed in the HV-1 region of D-loop mtDNA with a sequence length of 166 bp. The results revealed that the genetic distance between *Bos taurus*, *Bos inducus*, and *Bos javanicus* clusters reached 0.000-0.165 (Figure 3). HV-1 region of D-loop mtDNA (166 bp) was highly effective to classify three clusters of the cattle breed studied (Figure 4). Based on simulation results using the HV-2 region of D-loop mtDNA (755 bp), we found a difference in which the dendrogram showed inconsistency, compared to that from the HV-1 region; therefore, we recommended that clustering of cattle breeds including *Bos taurus, Bos inducus*, and *Bos javanicus* should be based on HV-1 region of D-loop mtDNA.

Furthermore, we also found that the

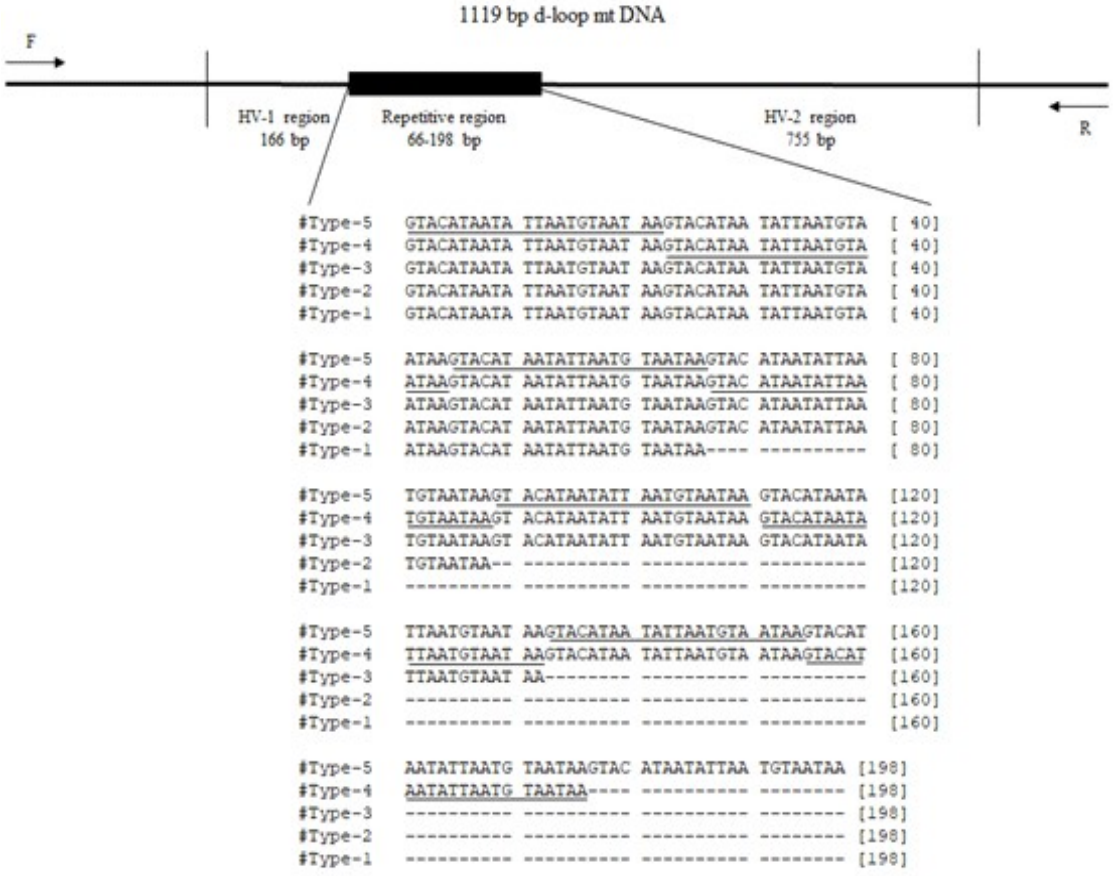

Figure 2. Structure of D-loop mtDNA and Pattern of Repetitive Nucleotide in Bali cattle.

Table 3. The Sequence of Repetitive Nucleotide in D-loop mtDNA of Bali Cattle

No.	Number and Pattern of Repetitive Sequence	Type	n	Frequency
1	$(GTACATAATATTAATGTAATAA)_3$	1	9	0.474
2	$(GTACATAATATTAATGTAATAA)_4$	2	2	0.105
3	$(GTACATAATATTAATGTAATAA)_6$	3	4	0.211
4	$(GTACATAATATTAATGTAATAA)_8$	4	2	0.105
5	$(GTACATAATATTAATGTAATAA)_9$	5	2	0.105
	Total		19	1.000

n is sample number

sequence of the HV-1 region in Bali cattle, SO cattle, and other cattle breeds used to determine genetic distance and genetic tree possessed 28 polymorphic sites (Figure 5). The polymorphic site at nucleotide 162 bp (15795 reference GenBank) was considered as a specific marker for *Bos javanicus*, *Bos indicus* and *Bos taurus,* namely adenine (A), guanine (G) and cytosine

Breed	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Simmental																													
Limousin	0.000																												
Angus	0.000	0.000																											
Ongole	0.024	0.024	0.024																										
Nellore	0.024	0.024	0.024	0.000																									
Sahiwal	0.024	0.024	0.024	0.000	0.000																								
SO-1	0.024	0.024	0.024	0.000	0.000	0.000																							
SO-2	0.024	0.024	0.024	0.000	0.000	0.000	0.000																						
SO-3	0.024	0.024	0.024	0.000	0.000	0.000	0.000	0.000																					
SO-4	0.024	0.024	0.024	0.000	0.000	0.000	0.000	0.000	0.000																				
SO-5	0.024	0.024	0.024	0.000	0.000	0.000	0.000	0.000	0.000	0.000																			
Bali_1	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152																		
Bali_2	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000																	
Bali_3	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000	0.000																
Bali_4	0.152	0.152	0.152	0.146	0.146	0.146	0.146	0.146	0.146	0.146	0.146	0.006	0.006	0.006															
Bali_5	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000	0.000	0.000	0.006														
Bali_6	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000	0.000	0.000	0.006	0.000													
Bali_7	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000	0.000	0.000	0.006	0.000	0.000												
Bali_8	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000	0.000	0.000	0.006	0.000	0.000	0.000											
Bali_9	0.152	0.152	0.152	0.146	0.146	0.146	0.146	0.146	0.146	0.146	0.146	0.006	0.006	0.006	0.000	0.006	0.006	0.006	0.006										
Bali_10	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.006									
Bali_11	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.006	0.000								
Bali_12	0.152	0.152	0.152	0.146	0.146	0.146	0.146	0.146	0.146	0.146	0.146	0.006	0.006	0.006	0.000	0.006	0.006	0.006	0.006	0.000	0.006	0.006							
Bali_13	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.006						
Bali_14	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.006	0.000					
Bali_15	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.006	0.000	0.000				
Bali_16	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.006	0.000	0.000	0.000			
Bali_17	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.006	0.000	0.000	0.000	0.000		
Bali_18	0.165	0.165	0.165	0.159	0.159	0.159	0.159	0.159	0.159	0.159	0.159	0.006	0.006	0.006	0.012	0.006	0.006	0.006	0.006	0.012	0.006	0.006	0.012	0.006	0.006	0.006	0.006	0.006	
Bali_19	0.159	0.159	0.159	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.000	0.006

Figure 3. The Genetic Distance of Bali Cattle, SO cattle, and Other Cattle Breeds Based on the HV-1 Region of D-loop mtDNA

(C). Meanwhile, the composition of A-T and G-C in the HV-1 region of D-loop mtDNA was 62.5% and 37.5%, suggesting no significant difference from the complete nucleotide composition of D-loop mtDNA in Bali cattle (921 bp) and SO cattle (913 bp) (Table 4). The composition of A-T and G-C in the repetitive sequence of D-loop mt DNA in Bali cattle was found 86.4% and 13.6% (Table 5). Hence, A-T in the repetitive sequence was more abundant than in the HV-1 region at a similar sequence of D-loop mt DNA. The difference in A-T composition, which is higher in repetitive nucleotide, caused instability of D-loop sequence, while also promoted variability among individuals; thus, this could be then utilized as a genetic marker primarily for Bali cattle due to its high polymorphism.

DISCUSSION

Variability of D-loop mtDNA in Bali Cattle and Sumba Ongole

The sequence length of D-loop mt DNA in Bali cattle in this present work varies from 921 to 1119 bp consisting of HV-1 region (166 bp), repetitive nucleotide (66-198 bp) and HV-2 (755 bp). In Bali cattle, variability in length of D-loop mt DNA was due to the presence of 22 bp nucleotide repeatedly present at 3-9 times, while it was also absent in sequence of D-loop mtDNA in SO cattle (913 bp) and other cattle breeds based on gene sequence analysis from GenBank. In the eukaryotic genome, the molecular marker was recognized as microsatellite comprising of repeated di-, tri-, and tetranucleotide (Viryanski, 2019) or 1-10 repeated nucleotide, and minisatellite consisting of 10-100 repeated nucleotide (Van Marle-Köster and Nel, 2003) or 6-100 repeated nucleotide (Vergnaud, 2002). Variability of repetitive nucleotide (22 bp) in D-loop mtDNA as found in Bali cattle can be employed as a DNA specific marker especially for understanding genetic diversity among individuals since they are known as polymorphic.

Bali cattle had higher genetic diversity than SO cattle in terms of allele number, the average number of nucleotide differences, number of polymorphic sites, and nucleotide variability, except for haplotype diversity which was higher in SO cattle than in Bali cattle. The number of

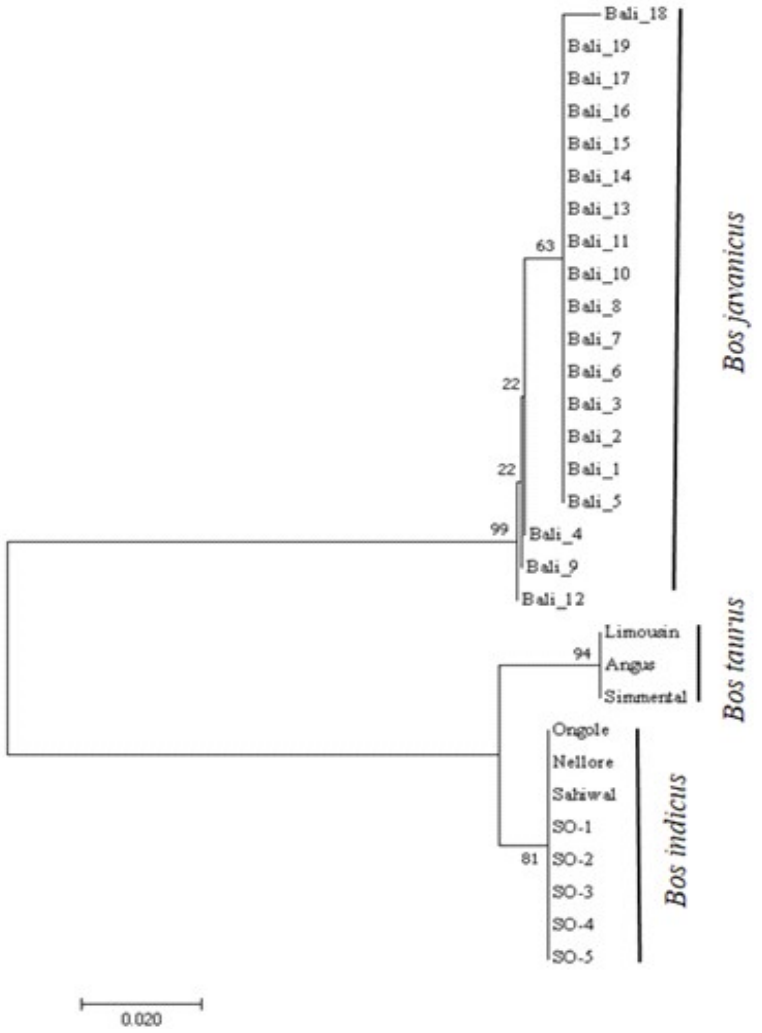

Figure 4. Genetic Tree of Bali Cattle, SO Cattle and Other Cattle Breeds using the Sequence of HV-1 Region of D-loop mtDNA Based on the NJ Method (1000 Bootstrap Replications)

haplotypes is a proper indicator of diversity derived from the maternal line (Ivankovic *et al*., 2014). Genetic diversity based on haplotype diversity (Hd) and nucleotide diversity (Pi) studied in sequence of D-loop mtDNA has been reported in 27 Podolian cattle breeds reaching 0.837 and 0.010 (Lorenzo *et al*., 2018), Brahman cattle reaching 0.978 and 0.0143 (Qu and Wu, 2006), Brangus-Ibage cattle reaching 0.581 and 0.009 (Henkes *et al*., 2005) as well as Chinese Wuchuan Black cattle reaching 0.909 and 0.055 (Yang *et al*., 2014). Our current investigation demonstrated that haplotype diversity and nucleotide diversity in Bali cattle reached 0.625 and 0.026, while SO cattle reached 0.900 and 0.006. Although the variability of haplotype and nucleotide seemed to be high in both cattle breeds, haplotype frequency was dominated by haplotype 2 (Hap-2), which might be associated with a reduction of haplotype (8 haplotypes) in Bali cattle (Table 2). The decline in haplotype number and genetic diversity for Bali cattle may relate to an artificial insemination program that has been intensively carried out, which in turn enables to eliminate of a particular haplotype as a specific marker of Bali cattle as a consequence of inbreeding pressure. Besides, the limited use of males for natural mating occurred as a result of inbreeding pressure, thereby alleviating the diversity of Bali cattle and SO cattle.

Genetic distance and genetic tree in Bali cattle and SO cattle

The results demonstrated cluster separation between Bali cattle (*Bos javanicus*) and SO cattle (*Bos indicus*), as well as other cattle breeds

Position (bp)/ Breed	223456667 2679053480	111111111 8022244455 7324501435	11111111] 55566667] 67802480]
#Simmental	ATAATTCCTA	CTCTAAGCAT	TACTCCGG
#Limousin	....	....	....
#Angus	....	....	....
#Ongole	..G.......	....	...CG.A.
#Nellore	..G.......	....	...CG.A.
#Sahiwal	..G.......	....	...CG.A.
#SO-1	..G.......	....	...CG.A.
#SO-2	..G.......	....	...CG.A.
#SO-3	..G.......	....	...CG.A.
#SO-4	..G.......	....	...CG.A.
#SO-5	..G.......	....	...CG.A.
#Bali_1	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_2	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_3	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_4	GCGGCCTT.G	TCTACC-TCC	CCTCAT.A
#Bali_5	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_6	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_7	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_8	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_9	GCGGCCTT.G	TCTACCCTCC	CCTCAT.A
#Bali_10	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_11	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_12	GCGGCCTT.G	TCTACC-TCC	CCTCAT.A
#Bali_13	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_14	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_15	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_16	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_17	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_18	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A
#Bali_19	GCGGCCTTCG	TCTACCCTCC	CCTCAT.A

Figure 5. Variability of sequence in HV-1 region of D-loop mtDNA.

including *Bos taurus*, using a sequence of D-loop mtDNA retrieved from GenBank. Previously, the clustering of cattle breeds based on genetic distance and the genetic tree was commonly performed using *Bos taurus* and *Bos inducus* (Chung, 2013; Xuan *et al*., 2010), since reference related to Bali cattle as *Bos javanicus* cluster has been scarcely available. Genetic diversity of cattle breeds could be researched not only according to partial fragment of D-loop mtDNA (Correia *et al*., 2017; Sari *et al*., 2016), complete fragment of d-loop mtDNA (Lorenzo *et al*., 2018), but also whole sequence of mtDNA genome (Pramod *et al*., 2018). Yet, it is noteworthy that the results of the analysis using entire fragments are expected to collect comprehensive relationships between individuals within species or among species closely related with divergence time (Rehman *et al*., 2017).

Our experimental results underline that Bali cattle is origin to Indonesia (Martojo, 2012), which is present in different cluster towards other cattle breeds in the world (Lenstra *et al*., 2014) including SO cattle as Indonesian local cattle imported from Nellore of India by Dutch in hundred years ago (Hartati *et al*., 2015). Hybridization of Bali cattle (*Bos javanicus*) with SO cattle (*Bos indicus*) formed Peranakan Ongole (PO) cattle that accounted for 6-7% of Bali cattle (Hartati *et al*., 2015), whereas Bali cattle contributed to Madura cattle (56%) and Galekan cattle 94% (Groeneveld *et al*., 2010). In summary, the existence of Bali cattle exhibited a remarkable contribution to the diversity of cattle breeds in Indonesia, according to current scientific evidence using D-loop mtDNA analysis (Agung and Hermansyah, 2018; Sutarno *et al*., 2015; Nijman *et al*., 2003).

CONCLUSION

The sequence of D-loop mtDNA in Bali cattle ranged from 921 to 1119 bp comprising of HV-1 region (166 bp), 22 bp repeated nucleotide (66-198 bp) and HV-2 region (755 bp), while SO cattle possessed a length of D-loop mtDNA

Table 4. Composition of Nucleotide in HV-1 Region of D-loop mtDNA

Breed	T(U)	C	A	G	Total
Simmental	22.1	25.0	43.6	9.3	172
Limousin	22.1	25.0	43.6	9.3	172
Angus	22.1	25.0	43.6	9.3	172
Ongole	21.5	25.0	43.6	9.9	172
Nellore	21.5	25.0	43.6	9.9	172
Sahiwal	21.5	25.0	43.6	9.9	172
S0-1	21.5	25.0	43.6	9.9	172
S0-2	21.5	25.0	43.6	9.9	172
S0-3	21.5	25.0	43.6	9.9	172
S0-4	21.5	25.0	43.6	9.9	172
S0-5	21.5	25.0	43.6	9.9	172
Bali-1	21.2	28.5	39.4	10.9	165
Bali-2	21.2	28.5	39.4	10.9	165
Bali-3	21.2	28.5	39.4	10.9	165
Bali-4	22.0	27.4	39.6	11.0	164
Bali-5	21.2	28.5	39.4	10.9	165
Bali-6	21.2	28.5	39.4	10.9	165
Bali-7	21.2	28.5	39.4	10.9	165
Bali-8	21.2	28.5	39.4	10.9	165
Bali-9	21.8	27.9	39.4	10.9	165
Bali-10	21.2	28.5	39.4	10.9	165
Bali-11	21.2	28.5	39.4	10.9	165
Bali-12	22.0	27.4	39.6	11.0	164
Bali-13	21.2	28.5	39.4	10.9	165
Bali-14	21.2	28.5	39.4	10.9	165
Bali-15	21.2	28.5	39.4	10.9	165
Bali-16	21.2	28.5	39.4	10.9	165
Bali-17	21.2	28.5	39.4	10.9	165
Bali-18	21.2	28.5	40.0	10.3	165
Bali-19	21.2	28.5	39.4	10.9	165
Average	21.5	27.1	41.0	10.4	167.5

Table 5. Composition of Nucleotide in the Repetitive Nucleotide of D-loop mtDNA in Bali Cattle

Breed	T(U)	C	A	G	Total
Bali-1	36 .4	4.5	50	9.1	198
Bali-2	36 .4	4.5	50.0	9.1	66
Bali-3	36 .4	4.5	50.0	9.1	132
Bali-4	36 .4	4.5	50.0	9.1	198
Bali-5	36 .4	4.5	50.0	9.1	66
Bali-6	36 .4	4.5	50.0	9.1	66
Bali-7	36 .4	4.5	50.0	9.1	176
Bali-8	36 .4	4.5	50.0	9.1	66
Bali-9	36 .4	4.5	50.0	9.1	176
Bali-10	36 .4	4.5	50.0	9.1	66
Bali-11	36 .4	4.5	50.0	9.1	132
Bali-12	36 .4	4.5	50.0	9.1	132
Bali-13	36 .4	4.5	50.0	9.1	66
Bali-14	36 .4	4.5	50.0	9.1	66
Bali-15	36 .4	4.5	50.0	9.1	66
Bali-16	36 .4	4.5	50.0	9.1	132
Bali-17	36 .4	4.5	50.0	9.1	88
Bali-18	36 .4	4.5	50.0	9.1	66
Bali-19	36 .4	4.5	50.0	9.1	88
Average	36 .4	4.5	50.0	9.1	107 .7

reaching 913 bp. Both cattle breeds were found to have high variability in haplotype and nucleotide, having 8 and 4 haplotypes, respectively. We also reported 5 types of repetitive nucleotide in Bali cattle, in which they demonstrated a high variability between individuals. Reconstruction of genetic tree based on HV-1 region of D-loop mtDNA (166 bp) could produce clear classification between Bali cattle as *Bos javanicus* cluster, SO cattle as *Bos indicus* cluster and other cattle breeds recorded as *Bos taurus* cluster with 28 polymorphic sites.

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