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Genetic diversity of Ongole Grade, Aceh, and Sumbawa cattle based on polymorphism on ND-5 fragment mitochondrial DNA using PCR-RFLP technique

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Abstract. *Sutarno, Zahrah S, Astirin OP, Herawati E, Setyawan AD. 2019. Genetic diversity of Ongole Grade, Aceh, and Sumbawa cattle based on polymorphism on ND-5 fragment mitochondrial DNA using PCR-RFLP technique. Biodiversitas 20: 783-788.* Genetic diversity is the basis of livestock breeding because it can be used as an initial improvement in livestock quality through artificial selection. This study aims to determine polymorphism in ND-5 fragment of mitochondrial DNA in Ongole Grade, Aceh, and Sumbawa cattle and their genetic diversity. The total DNA from the blood of the local cattle was extracted using the Wizard genomic DNA purification system from Promega and amplified using the PCR technique. The PCR product was then digested with *Hin*dIII enzyme using the RFLP technique to detect polymorphism. The genetic diversity of the Ongole Grade, Aceh, and Sumbawa cattle was analyzed using the formula from Nei and its genetic relationship was evaluated with the 2.02i NTSYSpc version program. Our findings showed there were polymorphisms in the ND-5 fragment of mitochondrial DNA. Digestion with *Hin*dIII restriction enzyme produces two types of haplotypes. Haplotype B is a 453 bp-sized DNA fragment that is not truncated by the *Hin*dIII enzyme, and haplotype A is a DNA fragment cut by *Hin*dIII enzyme into two with fragments of 336 bp and 117 bp. Polymorphism was found in Ongole Grade cattle, but not in Sumbawa and Aceh cattle. Haplotypes diversity in ND-5 fragments of mitochondrial DNA of Ongole Grade was 0.6250 while Sumbawa and Aceh cattle displayed no diversity of haplotypes. The genetic relationship shows that Sumbawa cattle belonged to the same cluster with Ongole Grade but separated from Aceh cattle.

Keywords: Genetic diversity, local Indonesian cattle, ND-5 fragment mitochondrial DNA, PCR-RFLP, polymorphism

INTRODUCTION

Indonesia has abundance and potentially great genetic diversity of local breed cattle that have been used as sources of meat, milk, labor, energy, and fertilizer. Among local Indonesian cattle, there are Aceh cattle in Nanggroe Aceh Darussalam, Pesisir cattle in West Sumatra, Java-Ongole cattle in Java, Madura cattle in Madura, and Bali cattle on Bali, Nusa Tenggara, and Sulawesi (Sutarno and Setyawan 2015, 2016).

Based on its genetic and phenotype characteristics, Bali cattle descend from a domestication process of wild banteng (*Bos javanicus*) (MacHugh 1996; Sutarno and Setyawan 2015) and displays minor changes on their physical traits compared to their ancestors (Handiwirawan and Subandriyo 2004; Sutarno and Setyawan 2016). Zebu cattle of Hissar variant was introduced from India to Sumbawa Island in 1908 and the offspring spread on this island, known as Sumbawa cattle (Decree of the Minister of Agriculture No. 2909/Kpts/OT.140/6/2011). Meanwhile, Ongole Grade cattle (*Sapi Peranakan Ongole*) is the result of a cross breeding from another Indian zebu cattle, namely Ongole cattle which originally entered Sumba Island in 1905 and spread to Java through the "Ongolization" program. The program has led to a generation of Ongole Grade cattle and other cross-bred cattle and eventually contributed to the loss of previously Java cattle (Sutarno and Setyawan 2015, 2016).

As for local Indonesian cattle, Ongole Grade, Aceh, and Sumbawa cattle are better suited to environmental resilience. The females recover quickly after calving, the males have a good quality of cement, and they are all generally stronger than imported cattle. Nevertheless, due to their lower quality and quantity of production, the Indonesian farmers, especially with large business capital, prefer to raise imported cattle (Sutarno 1998), especially European cattle such as Simmental and Limousin for meat as well as Friesian-Holstein for milk (Sutarno and Setyawan 2015, 2016). This trend causes genetic diversity in cattle to decline very rapidly (Hall and Bradley 1995; Hammond and Leitch 1995). In fact, genetic diversity is the basis of cross-breeding for livestock (Buis et al. 1994) because this information can be used as a starting point to increase the quantity and quality of species through artificial selection and becomes the initial knowledge

needed in conservation of genetic resources (Kidd et al. 2002).

The decline in local cattle production has led to higher imports of meat and cattle to meet domestic needs. The Indonesian Ministry of Agriculture demand to further stimulate the development of local cattle in an attempt to reduce the dependency of imported cattle. Increasing the quality and quantity of local beef production will be more appropriate if it is conducted through selection that is not only based on the phenotype, but also through the direct selection at the DNA level which encodes the targeted phenotype. Bovine genome mapping based on markers on DNA levels using molecular techniques such as Microsatellite, RAPD, RFLP PCR, has made it possible to identify gene loci that are responsible for variations in characteristics that have important economic value (QTL) (Sutarno et al. 2005; Sutarno et al. 2018).

Mitochondrial DNA is widely used to express genetic variation (Loftus et al. 1994), because of its relatively small size, is involved in energy synthesis and has mutation rate at 5-10 times higher than core DNA (Lindberg 1989; Solihin 1994). Polymorphism of mitochondrial DNA is known to influence the phenotype (Wallace et al. 1995) and the production characteristic (Lindberg 1989; Ron et al. 1993; Schutz et al. 1993; Sutarno et al. 2002). Previous studies have shown that mitochondrial DNA affects growth, reproduction and the nature of production in livestock (Schutz et al. 1993). Growth is influenced by two main factors, namely the hormonal system and energy availability. Mitochondria are the center of energy synthesis in cells and control the total production of energy in the body. Various types of enzymes are involved in the synthesis of energy, and some of these enzymes are encoded by mitochondrial DNA. One of the genes that encode the enzyme involved in energy synthesis is known as ND-5 (Lelana et al. 2003).

Identification on the ND-5 fragments of mitochondrial DNA in Bengali cattle (Ongole Grade) has been carried out by Lelana et al. (2003). By applying *Hin*dIII restriction enzyme, the study showed that polymorphism (genetic variation) was found in ND-5 fragments of mitochondrial DNA in Bengali cattle (Ongole Grade) and Madura cattle, yet no information is available so far regarding polymorphism on ND-5 fragments in Aceh and Sumbawa cattle. Therefore, the identification of polymorphisms on ND-5 mitochondrial DNA in these cattle needs to be carried out.

Polymorphism is expressed by genes and can be used to analyze the genetic state of a population. In one population, polymorphism is determined by loci that exhibit high values of heterozygosity. Polymorphism analysis of ND-5 fragments in mitochondrial DNA from Ongole Grade, Aceh, and Sumbawa cattle allow us to asses the genotypic distribution of those cattle. Thus, it can be applied simultaneously as a Marker Assisted Selection (MAS) to select the production characteristic with high economic value as a basis for genetic selection in breeding programs to improve the quality and quantity of cattle production.

The objectives of this study were (i) to study the polymorphism of ND-5 fragments in mitochondrial DNA among Ongole Grade, Aceh, and Sumbawa cattle. (ii) to study the diversity of haplotypes of Ongole Grade, Aceh, and Sumbawa cattle.

MATERIALS AND METHODS

Materials

Blood samples were taken from 12 individuals in each cattle breed. The Ongole Grade cattle were obtained from the farmers in Wonogiri, Central Java, Indonesia, whereas Aceh and Sumbawa cattle were obtained from Superior Animal Breeding Center and Animal Feed, Indrapuri, Aceh Besar, Indonesia and farmers in Dompu, West Nusa Tenggara, Indonesia, respectively.

Methods

Blood samples and mitochondrial DNA extraction

Blood samples (5 mL) were taken from each cattle using a 10 mL venoject tube containing lithium heparin. Some amount of the fresh blood was analyzed directly, and the remaining was stored at -20°C for reference. DNA isolation from blood samples was done using Wizard Genomic DNA Purification Kit (Promega) according to manufacturer instruction. Briefly, 450 µL Cell Lysis Solution was added into 300 µL total blood, mixed thoroughly by inverting the tube 5-6 times, then incubate for 10 minutes at room temperature (invert 2-3 times once during the incubation) to lyse the red blood cells. The mixture was then centrifuged at 14,000×*g* for 20 seconds at room temperature to obtain pellet containing white blood cells. The supernatant was removed and discarded, followed by vortexing the tube for 3-5 minutes to separate clumps of white blood cells. Nuclei Lysis Solution (150 µL) was added to the tube containing the resuspended cells and mixed well by gently pipetting the solution 5-6 times to lyse the white blood cells. RNase Solution (1.5 µL) was mixed to the nuclear lysate, incubated at 37°C for 15 minutes, and then cool to room temperature. Protein Precipitation Solution (60 µL) was added to the Bio lysate, vortex vigorously for 10-20 seconds, then centrifuged at 14,000×*g* for 3 minutes at room temperature. At this step, a dark brown protein pellet should be visible. The supernatant was transferred to a clean 1.5 mL microcentrifuge tube containing 150 µL of room-temperature isopropanol. The solution was gently mixed by inversion until the white thread-like strands of DNA form a visible mass. DNA was centrifuged at 14,000×*g* for 1 minute at room temperature. The DNA will be visible as a small white pellet. Supernatant was removed by decantation, added with one sample volume (300 µL) of room-temperature 70% ethanol to the DNA. The tube was inverted several times gently to wash the DNA pellet and the sides of the microcentrifuge tube, followed by centrifuge again at 14,000×*g* for 1 minute at room temperature. After removing the ethanol, the tube was inverted on clean absorbent paper to let the pellet air-dry, then the DNA was resuspended in Rehydration Solution (100 µL).

PCR reaction

The results of DNA extraction were used for PCR reactions carried out in GeneAmp PCR System 2400 Thermo Cycler (Perkin Elmer). This reaction amplified mitochondrial DNA in the ND-5 region. The reaction was carried out in a mixed volume of 25 µL containing 12 µL Go Taq (Promega), 5 µL DNA template, ND-L primer and ND-R 1 µL each, 6 µL nuclear-free water in a 0.6 mL PCR tube. Below are primers used for ND-5 amplification:

ND-L: 5'- ATCCGTTGGTCT TAGGAACC-3'
ND-R: 5'- TTGCGGTTACAAGGATGAGC-3'

The PCR condition was set as follows: initial denaturation at 94°C; 5 min (1 cycle), 30 cycles of amplification consisting of three stages: (i) denaturation at 94°C; 45 s, (ii) 58°C; 45 s, and (iii) 72°C; 1 min, followed by 1 cycle of final polymerization at 72°C; 6 minutes (Lelana et al. 2003).

DNA digestion and electrophoresis

DNA fragments from the PCR product (10 µL) were digested using *Hin*dIII (ThermoFisher Scientific) restriction enzyme (1 µL) in a mixture of reaction buffer and nuclear-free water. The digested DNA was separated in a 1% agarose gel loaded in a horizontal electrophoresis chamber. After electrophoresis, DNA was visualized under UV-lamp and documented under Gel Doc 2000 (Bio-Rad) by using a red filter.

RFLP analysis

Genetic diversity at mitochondrial DNA loci was analyzed using the below equation (Nei 1973, 1975):

$H = 1 - J$, and $J = (A^2 + B^2)$

Where:
H: haplotype diversity
A: frequency of A haplotype
B: frequency of B haplotype

To examine the genetic relationship (similarity) between individuals, we used the similarity index (Is) with the NTSYSpc 2.02i master program (Yeh et al. 1999).

RESULTS AND DISCUSSION

Polymorphism analysis of ND-5 fragments in mitochondrial DNA was performed in three stages, namely: DNA extraction, DNA amplification by PCR and targeted-DNA Digestion with restriction enzymes. The success of amplification of ND-5 mitochondrial DNA fragments is determined mainly by the precise attachment of primer pair onto DNA template as well as the overall PCR condition. In this study, we successfully amplified a specific band, as indicated by the formation of a single band at expected size (453 bp) (Figure 1). The band intensity varied between samples, suggesting a different concentration of PCR product.

The PCR products were then incubated with *Hin*dIII restriction enzymes for 20 minutes at 37°C. The incubation temperature refers to the temperature at which *Hin*dIII enzymes can work optimally so that the PCR product can be cut off entirely. Likewise, inappropriate incubation temperatures may cause incomplete DNA cutting due to low enzyme specificity. The result of PCR product digestion was then loaded into 1% electrophoresis gel (Figure 2). The result shows two kinds of haplotype patterns, namely A haplotype which is a commonly found haplotype (truncated haplotype at two sites) and B haplotype which is a rare haplotype (uncut haplotype).

A Haplotype forms two DNA bands measuring 336 bp and 117 bp, while B haplotype only forms one size of DNA band at 453 bp (Figure 2). This measurement referred to the size of the mitochondrial DNA sequence previously published by Anderson et al. (1982) and Sutarno et al. (2002). The ND-5 fragment lies in the position between bases 12058 and 12510 and has a *Hin*dIII restriction site at base position 12174. When the ND-5 fragment is digested using *Hin*dIII restriction enzyme, two fragments will be produced consisting of 117 and 336 bp (Sutarno et al. 2002) (Table 1).

Both haplotype patterns show the genetic variation among Ongole Grade, Aceh, and Sumbawa cattle, although they exhibited different frequency (Table 2).

Variation is a common characteristic found in a population. Diversity occurs not only between family but also within a family, between any population, and among individuals (Handiwirawan et al. 1998). Based on the analysis on 12 individuals of cattle performed in this study, the variation occurred only in Ongole Grade as indicated by the presence of A and B haplotypes (Figure 3). Meanwhile, the population of Sumbawa cattle harbors only A haplotype, whereas Sumbawa cattle solely has B haplotypes (Figure 4-5).

Table 1. Restriction site produced by *Hin*dIII enzymatic digestion on ND-5 fragments of mitochondrial DNA

Enzyme	Allele (Haplotype)	Number of the restriction site	Fragment size (kb)
*Hin*dIII	A	1	0.336
			0.117
	B	0	0.453

Table 2. Frequency of haplotypes of Ongole Grade, Sumbawa, and Aceh cattle

Cattle breed	Haplotype	
	A	B
Ongole Grade	0.75	0.25
Sumbawa	1.00	0.00
Aceh	0.00	1.00

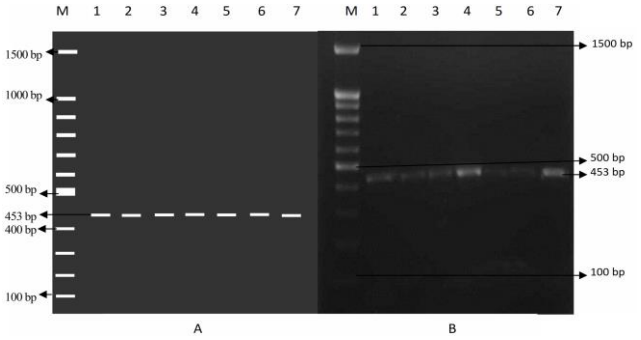

Figure 1. Agarose gel electrophoresis showing the specificity of PCR-amplified ND-5 fragments. PCR product size is 453 bp. A: Schematic drawing of electrophoresis in B; B: Agarose gel electrophoresis; M: Marker; 1, 2, 3: Aceh cattle; 4, 5: Ongole Grade cattle; 6, 7: Sumbawa cattle

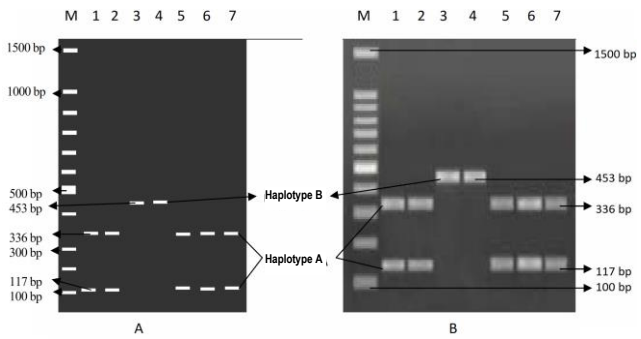

Figure 2. Agarose gel electrophoresis showing a variation on ND-5 fragments of mitochondrial DNA detected by PCR-RFLP using *Hin*dIII enzyme. Lane 1, 2, 5, 6, 7: ND-5 fragments of mitochondrial DNA digested using *Hin*dIII (A haplotype), lane 3, 4: uncut ND-5 fragments of mitochondrial DNA (B haplotype). A: Schematic drawing of electrophoresis in B; B: Agarose gel electrophoresis; M: Marker; 1, 2, 3: Aceh cattle; 4, 5: Ongole Grade cattle; 6, 7: Sumbawa cattle

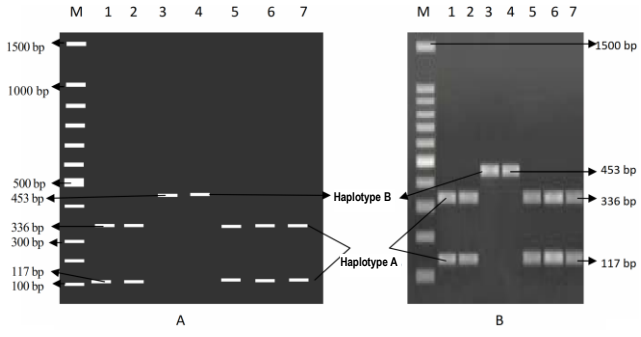

Figure 3. Electrophoresis of DNA from Ongole Grade cattle showing variation in ND-% fragment of mitochondrial DNA detected by using PCR-RFLP technique with *Hin*dIII restriction enzyme. A: Schematic drawing of electrophoresis in B; B: Agarose gel electrophoresis. Lane 1, 2, 5, 6, 7: ND-5 fragment of mitochondrial DNA digested with *Hin*dIII (A haplotype), Lane 3, 4: undigested ND-5 fragment of mitochondrial DNA (B haplotype)

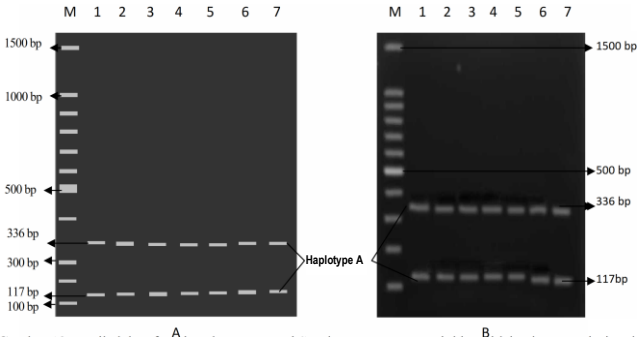

Figure 4. Electrophoresis of DNA from Sumbawa cattle shows no variation on the ND-5 fragments of mitochondrial DNA. ND-5 fragments were entirely digested by *Hin*dIII (A haplotype). A: Schematic drawing of electrophoresis in B; B: Agarose gel electrophoresis

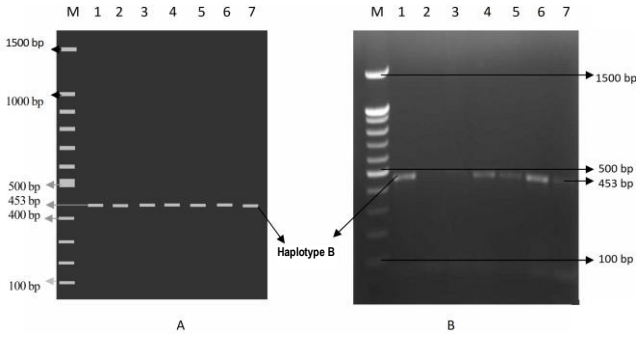

Figure 5. Electrophoresis of DNA from Sumbawa cattle shows no variation on the ND-5 fragments of mitochondrial DNA. ND-5 fragments were entirely digested by *Hin*dIII (A haplotype). A: Schematic drawing of electrophoresis in B; B: Agarose gel electrophoresis

The frequency of haplotype shows the occurrence of each haplotype of the sample under study. A haplotype occurred most frequently in Sumbawa cattle which was equal to 1.00, but not exist in Aceh cattle. On the contrary, B haplotypes are most abundant in Aceh cattle, but least appeared in Sumbawa cattle. Ongole Grade cattle possessed both haplotypes by 0.75 of A haplotype and 0.25 of B haplotype. Variations that occur in ND-5 fragments of mitochondrial DNA may be caused by the loss of the *Hin*dIII restriction site due to mutations, natural selection, as well as breeding. The haplotype frequency was further used for calculating the genetic diversity in the three cattle aforementioned (Table 3).

Table 3. Genetic diversity of Ongole Grade, Sumbawa, and Aceh cattle

Cattle breed	Haplotype diversity
Ongole Grade	0.6250
Sumbawa	0.0000
Aceh	0.0000

Genetic diversity of haplotypes was shown only in Ongole Grade cattle (0.6250) (Table 3). Genetic diversity was calculated from the diversity index of ND-5 fragments of mitochondrial DNA in the cattle population studied. An earlier study has measured that diversity of haplotypes in Bengali cattle (Ongole Grade) was 0.2112 (Lelana et al. 2003). This data suggests that there is an increase in the diversity of haplotypes in Ongole Breeds. The high or low genetic variation in the cattle population might be caused by the rate of mating, migration, and selection. When the frequency of inbreeding is high, there will be a limitation of gene exchange across species, thus contributing to lower genetic diversity, and *vice versa*. Migration causes great genetic exchange between individuals that breed in a population; hence, many variations in the genetic background arise (the resulting genes vary). In contrast, selection led to decrease in variation in a population because gene exchange will be limited. This mechanism is likely to occur in the population of Aceh and Sumbawa cattle.

The number of genetic diversity in the local population varies greatly. Two main factors responsible for this variation are the reproduction method (sexual or asexual) and population size. Also, the amount of genetic diversity within a species depends on the number of individuals, geographic distribution, the level of isolation from the population and its genetic system. The process of natural and anthropogenic selection carry out an important role as well as factors that influence spatial and temporal changes in the genetic composition of species or populations (Saubari 2010).

Measurement of genetic diversity at the haplotype level is thought to be sufficient to study polymorphisms at the DNA level. The study of genetic variation is more accurate than the study of proteins because changes in the constituents of nucleic acid base do not necessarily change the protein products produced as expressions of genes, thereby DNA variation is not necessarily indicated by the variation of proteins (Sutarno et al. 2002).

Variations in ND-5 fragments of mitochondrial DNA may occur by mutations, natural selection, and breeding. Mutations are changes in the arrangement of an individual's genes which include a change in nucleotide substances, insertions, and deletions (Sutarno 1998). This process is a determinant of genetic variation and is triggered by chemicals, physical stimulation such as high temperatures and UV radiation. Mutations in one nucleotide, for example, can lead to variations in the structure or sequence of nucleotides of a gene that gives rise to a new pattern if the DNA is cut with a restriction enzyme and then electrophoresed. The mutation may cause loss or generation of a new restriction site.

The relationship within a species and between species is described by a phylogenetic tree and its genetic distance. The phylogenetic tree is a branching diagram consisting of points as representatives of taxonomic units and branches (which are the connecting paths for these points). Phylogenetic tree describes the evolutionary relationship and topology between organisms or populations (Avise 1994). We obtained genetic distance based on the polymorphism of the ND-5 fragments of mitochondrial DNA as displayed in Figure 6.

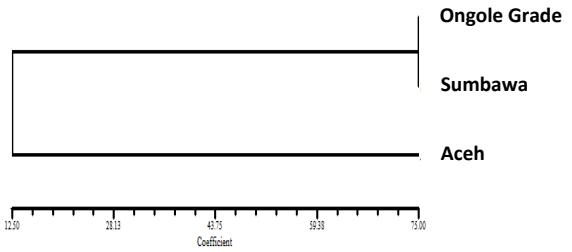

Figure 6. Genetic distance of Ongole Grade, Sumbawa, and Aceh cattle based on polymorphism of ND-5 fragments of mitochondrial DNA

Here, two clusters are generated. The first cluster was formed between Ongole Grade and Sumbawa cattle, indicating that both had the closest relationship with a coefficient of similarity index of 75%. The second cluster was formed between the two types of cattle, Ongole Grade/Sumbawa and Aceh cattle with a coefficient of similarity index of 12.5% (Figure 6).

Based on analysis on the D-Loop fragment of cattle mitochondrial DNA, Aceh cattle are closely related to Sumatra Pesisir cattle and the later formed one cluster with Ongole Grade cattle, whereas in other clusters there are Bali and Madura cattle (Abdullah 2008). The result differences with our study may be attributed to maternal differences from the parent of each cattle studied considering that mitochondrial DNA is maternal DNA.

Ongole Grade cattle descend from cross breeding of the zebu cattle, while Sumbawa cattle are Bali cattle bred on Sumbawa. Given that Bali cattle are the result of the domination of wild cattle, Sumbawa cattle carry many genes from wild cattle. Aceh cattle are the result of matting between Sumatra wild cattle and zebu cattle. Our findings showed that the Ongole Breeders were more closely related to Sumbawa cattle compared to Aceh cattle which initially contained the zebu gene. This phenomenon could be due to the occurrence of natural mating because the livestock system in Sumbawa employed a loose livestock system where cattle were released in the open ground for several months and then grazed again, so there is a possibility that the Sumbawa cattle mate naturally with zebu cattle. In addition to natural matting, the phenomenon could be due to the fact that Bali cattle which are the domestication of the wild cattle are genetically no longer pure. In line with the previous study, Bali cattle showed diversity in their blood hemoglobin (Noor et al. 2001). A pure Bali cattle only possess globin βxbali. However, among 8 samples of Bali cattle examined, 3 samples were from Sumbawa and 3 samples were from Bali, showed that 2 Bali cattle possessed globin βxbali and βB and βA2 which exist in Brangus cattle. Whereas in 2 samples of Bali cattle originating from Sumbawa, it contained globin βxbali, βA1, and βA2 in one cattle and the other did not have globin βxbali but βA1 and βA2 which exist in Limousin, Simmental, and Brangus cattle.

We performed tracing of genetic relationship using mitochondrial DNA based on haploid maternal inheritance patterns and hypervariability of ND-5 regions; thus, it is

likely that the Sumbawa and Ongole Grade cattle were derived from the female parent of the zebu while Aceh cattle originated from the female parent of the wild cattle. Knowledge of genetic variation of livestock is useful for cross breeder and genetic application. It can be used for animal identification, genealogical analysis, gene mapping, as well as markers of desired traits. All properties that appear to be influenced by genetic information are carried by DNA, so that DNA variations are associated with phenotypic variations. Therefore, genetic variation can be used as a basis for animal selection through a technique known as Marker Assisted Selection (MAS) or selection based on gene markers. Genetic variations can also be used for species conservation in animals (Sutarno et al. 2005).

In conclusion, polymorphism in ND-5 fragments of mitochondrial DNA was found in Ongole Grade showing two types of haplotypes, namely A and B haplotypes. On the other hand, Sumbawa and Aceh cattle did not show polymorphisms as evidenced by the formation of only A haplotype or B haplotype in Sumbawa and Aceh cattle respectively. The haplotype diversity of Ongole Grade was 0.6250 while Sumbawa and Aceh cattle showed no diversity of haplotypes.

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