

## Haplotype Diversity of Partial Cytochrome b Gene in Kebumen Ongole Grade Cattle

T. Hartatik\*, D. Maharani, J. H. P. Sidadolog, A. Fathoni, & Sumadi

Laboratory of Animal Genetic and Breeding, Department of Animal Breeding and Reproduction,  
Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia

\*Email of corresponding author: [tety@ugm.ac.id](mailto:tety@ugm.ac.id)

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### ABSTRACT

Cytochrome b gene is commonly used to figure out the genetic variation in animals. This research was designed to explore specific markers of Kebumen Ongole grade cattle and to understand the relationship between Kebumen and Brahman cattle using mtDNA *cyt b* sequences. The sequence of mtDNA cytochrome b gene of 14 individuals Kebumen Ongole grade cattle and 15 individuals Brahman cattle was determined by using polymerase chain reaction and sequencing with a pair of primer. The haploview software was utilized to linkage disequilibrium (LD) analysis. As a result, 36 SNPs (single nucleotide polymorphisms) with 8 haplotypes were identified in Kebumen Ongole grade cattle. In contrast, a small number of SNPs (7) and haplotypes (6) were observed in Brahman cattle. The homozygosity in Brahman cattle was higher than that in Kebumen Ongole grade cattle. Moreover, based on mtDNA sequences, 50% of Kebumen Ongole grade cattle had 85% similarity with Brahman cattle. The SNP number and LD values of Kebumen Ongole grade cattle were greater than those of Brahman cattle. It was presumed that Brahman cattle might be introduced into Kebumen territory. In conclusion, some of Kebumen Ongole grade cattle have genetic relation with Brahman cattle. In addition, the information of SNP and haplotype results from this research can be used as markers to differentiate breeds of animals.

**Keywords:** *haplotype, cyt b gene, kebumen ongole grade cattle, linkage disequilibrium*

### INTRODUCTION

The genetic diversity of cattle in Indonesia was established as a result of crossbreeding and upgrading since 1930s. Historically, Ongole grade cattle was the result of crossing between Javanese cattle (*Bos javanicus*) and Ongole cattle (*Bos indicus*). Ongole grade cattle has a good productivity and resistance to tropical environments. This cattle has a white-gray skin, long tail, black fur around the eyes, short head, short horns, long straight ears, and a rather large belly (Sutarno *et al.*, 2015). Ongole grade cattle in Indonesia is known with the name of Peranakan Ongole (PO). PO cattle is the result of a cross between Java and Sumba Ongole cattle (Suyadi *et al.*, 2014). At present, many breeders cross them with Brahman cattle.

Brahman cattle, originally from Indian *Bos indicus*, firstly imported into the US since 1849 and it has been crossed with many new breeds. Brahman cattle has a long large body, big shoulders, loose skin, many folds in the lower jaw to the front of chest, longways head, large hanging ears, and large thighs (Sutarno *et al.*, 2015). Brahman cattles were imported into Indonesia in 2006 and distributed to the most areas of Indonesia for supporting beef cattle programe. They were crossed

with Ongole grade cattle to produce livestock which have a good productivity and a good adaptability (Sutarno *et al.*, 2015).

In Kebumen, breeding programe to improve genetic quality of PO cattle had been done before 1930 and they look like a pure Ongole cattle (Utomo *et al.*, 2015). Based on physical observation in the field, we found the similarities between Kebumen Ongole grade cattle and Brahman cattle. Kebumen Ongole grade cattle has a bigger body size compared with Indonesian National Standard for Ongole cattle both males and females in other areas. The qualitative characteristics of Kebumen Ongole grade cattle are white body color, black muzzle, black or red vulva, lateral triangle head, long horns, long wattle, and big hump (Sudrajad and Subiharta, 2014). Their population in the District of Kebumen in 2014 was 80,486 heads. They were declared as a local Kebumen cattle strain through the Decision Letter of Indonesian Minister of Agriculture no. 358/Kpts/PK. 040/6/2015 on 8 June 2015.

Based on available records, it was established that Brahman cattle was crossed with Ongole grade cattle in Kebumen, so we proposed that Kebumen Ongole grade cattle and Brahman cattle had blood relationship. Finding out the relationship between both Kebumen

Ongole grade cattle and Brahman cattle could be through a molecular techniques, one of them was Mitochondrial DNA analysis. Mitochondrial DNA can be used as a marker for the study of animal domestication (Stock *et al.*, 2009). The use of PCR of mitochondrial DNA (mtDNA) is a recent method to identify animal species (Minarovič, *et al.*, 2010). Our previous study (Hartatik *et al.*, 2015) reported that mitochondrial cytochrome b (*Cyt b*) gene fragment also could be used for identification of species origin by the PCR method. The result of PCR showed two different fragments in two species between cattle (472 bp) and buffalo (124 bp) (Munira *et al.*, 2016).

Genetic marker of *cyt b* genes can be derived from the maternal line and used to determine the origin of a cattle. Romaino *et al.* (2014) reported that three Malaysian cattle breeds could be distinguished into wild cattle and domestic cattle by the amplification of the *Cyt b* gene. Several studies on mtDNAs in local cattle of Indonesia have been reported. For examples, local breeds including both Jabres and Rambon cattle in Brebes and Bondowoso Regency, respectively, are similar with Zebu and Bali cattle (Sutarno and Setyawan, 2015). Other results based on mtDNA *cyt b* marker showed that there were two haplotypes (A and B) of local cattle in Indonesia (Hartatik *et al.*, 2015). Those result showed that both Aceh and Pesisir cattle have haplotype B, while Bali cattle and Limura cattle (cross between Limousin bull and Madura cattle by artificial insemination) have different haplotype (A). However, Madura cattle and Java cattle have both haplotypes (A and B).

Studies using mtDNA can also be connected with development of high-throughput technology. One of the method that can be used for a genome-wide analysis of the non-random connection alleles at different loci named as linkage disequilibrium (LD) (Jasielczuk *et al.*, 2016, Niu *et al.*, 2016). The exploration of LD also can provide important insights for guiding selection in the genomic levels (Brito *et al.*, 2011; Zhou *et al.*, 2013). Previous studies proved that LD levels had effect on the accuracy of genomic prediction (Yang *et al.*, 2012; Cuyabano *et al.*, 2014).

Since there was no study about *cyt b* gene as DNA markers in Kebumen Ongole grade cattle, this research was designed to explore specific markers of Kebumen Ongole grade cattle and to understand the relationship between Kebumen and Brahman cattle using mtDNA *cyt b* sequences. This study could contribute to the understanding of genetic variation of another local cattle in Indonesia.

## MATERIALS AND METHODS

### Samples and DNA Extraction

Twenty-nine local cattle were used in the study consisting of Kebumen Ongole grade cattle (n= 14 heads) and Brahman cattle (n= 15 heads) as a control. Brahman cattle were used as controls because we wanted to understand the DNA composition of Brahman cattle in Kebumen Ongole grade cattle. Blood samples of Kebumen Ongole grade cattle were collected based on

their phenotypic characteristics which were close to that of Brahman cattle. Blood samples of Brahman cattle was collected from BPTUHPT Sembawa (Lampung). All the blood samples used in this study were taken from female cattle. The blood samples were collected from all animals from jugular vein with 22½G needle and collected in K3EDTA tubes, then the collected samples were stored at -20°C. Then, about 3 mL of blood samples were also collected for genomic DNA isolation using gSYNC™DNA Extraction Kit (Genetika Science).

### Polymerase Chain Reaction (PCR)

The amplification of DNA samples in this study was conducted by following the method modified by Hartatik *et al.* (2015). The primers used in this study were forward 5'-AAAAACCACCGTTGTTATTCAACTA-3' and reverse 5'-GCCCTCAGAATGATATTTGTCTCA3' (Wolf *et al.*, 1999). The total volume of 30 µL of PCR mixture were prepared and amplified by using the program: pre-denaturation for 2 min at 94 °C, denaturation at 95 °C for 36 s, annealing at 51 °C for 73 s, extension at 72 °C for 84 s, and final extension at 72 °C for 35 cycles for 3 min by Primus-25 Advanced (Germany) Thermal Cycler machine. The PCR products were visualized by 1% agarose gel electrophoresis under UV light. The expected product size of cytochrome b gene was 464 bp according to reference GenBank Acc.No. JN817304, *Bos indicus* (Figure 1A).

### Sequencing Analysis

PCR products with a total volume of 30 µL from each sample were sent to the 1st Base Genetic Science. Sequencing process used BigDye® Terminator v3.1 Cycle Sequencing Kit with the machine of ABI PRISM 3730xl Genetic Analyzer developed by Applied Biosystems, USA. One direct sequence analysis was carried out by using the forward primer of *cyt b* gene. The results of the sequencing were then analyzed by using Bioedit 7.5 to determine the SNP and haplotype from both groups of cattle.

### Linkage Disequilibrium Analysis

Relationships between the SNPs to be recommended to form haplotype were estimated using LD analysis by haploview software version 4.2 (Broad Institute). Recommended haplotype pairs were the pairs having the values of  $D'$  and  $R^2$  above 75%. Haploview software was used in this study by following method modified by Barret *et al.* (2005).

## RESULTS

### Sequencing Analysis

The result of PCR product size in 29 samples were medium to high intensity. The results of PCR 464 bp with the flanking primer of cytochrome b gene can be seen in Figure 1. Further analysis using PCR product

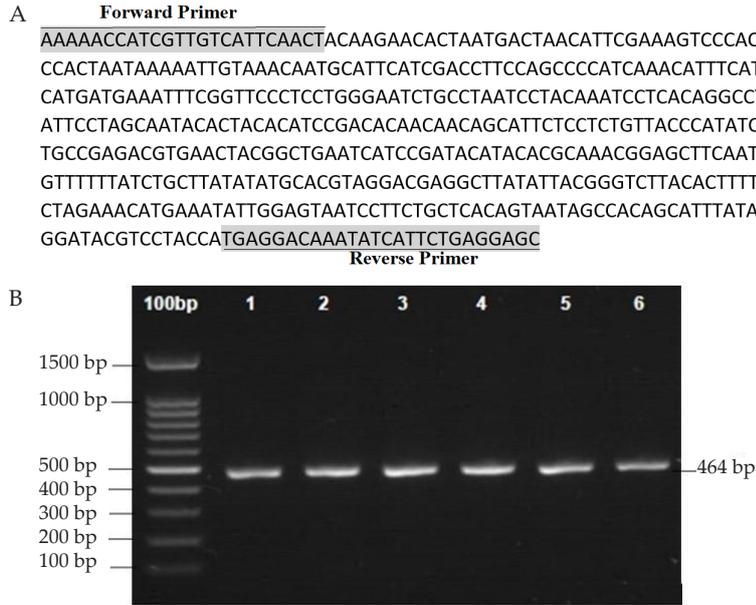


Figure 1. The predicted sequence result (A) and 464 bp product size PCR of Cyt-b gene (B)

Sample	Single Nucleotide Polymorphism (1-36)																																				H	
	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6		
CP1	3	4	4	2	3	3	2	1	3	2	1	4	2	4	4	3	3	4	4	4	4	4	4	3	3	3	4	3	3	3	4	1	4	4	4	4	2	I
Cp7	3	4	4	2	3	3	2	1	3	2	1	4	2	4	4	3	3	4	4	4	4	4	4	3	3	3	4	3	3	3	4	1	4	4	4	4	2	I
Cp2	3	4	4	2	3	3	2	1	3	2	1	4	2	4	4	3	3	4	4	4	4	4	4	3	3	3	4	3	3	3	4	1	4	4	4	4	4	II
Cp4	3	4	4	2	3	3	2	1	3	2	1	4	2	4	4	3	3	4	4	4	4	4	4	3	3	3	4	3	3	3	4	1	4	4	4	4	4	II
Cp5	3	4	4	2	3	3	2	1	3	2	1	4	2	4	4	3	3	4	4	4	4	4	4	3	3	3	4	3	3	3	4	1	4	4	4	4	4	II
Cp8	3	4	4	2	3	3	2	1	3	2	1	4	2	4	4	3	3	4	4	4	4	4	4	3	3	3	4	3	3	3	4	1	4	4	4	4	4	II
Cp11	3	4	4	2	3	3	2	1	3	2	1	4	2	4	4	3	3	4	4	4	4	4	4	3	3	3	4	3	3	3	4	1	4	4	4	3	4	III
Cp13	3	4	4	2	3	3	2	1	3	2	1	4	2	4	4	3	3	4	3	4	4	4	4	3	3	3	4	3	3	3	4	1	4	4	4	4	2	IV
Cp14	4	3	3	1	4	4	1	1	4	1	3	3	1	3	3	4	4	3	4	3	3	3	3	4	4	3	4	4	4	4	4	2	3	3	3	4	4	V
Cp3	4	3	3	1	4	4	1	2	4	1	3	3	1	3	3	4	4	3	4	3	3	3	3	4	4	4	4	4	4	4	3	2	3	3	3	4	4	VI
Cp10	4	3	3	1	4	4	1	2	4	1	3	3	1	3	3	4	4	3	4	3	3	3	3	4	4	4	4	4	4	4	3	2	3	3	3	4	4	VI
Cp12	4	3	3	1	4	4	1	2	4	1	3	3	1	3	3	4	4	3	4	3	3	3	4	4	4	4	4	4	4	3	2	3	3	3	4	4	VI	
Cp6	4	3	3	1	4	4	1	2	4	1	3	3	1	3	3	4	4	3	4	3	3	3	4	4	4	4	4	4	4	3	2	3	3	3	4	2	VII	
Cp9	4	3	3	1	4	4	1	2	4	1	3	3	1	3	3	4	4	3	4	3	3	3	4	4	4	4	4	4	3	2	3	3	3	4	4	VIII		

Figure 2. Identification of haplotype marker based on Cyt b gen in Kebumen Ongole grade cattle

was direct sequencing by one site of forward primer. The results of sequencing analysis showed 36 SNPs with 8 haplotypes in Kebumen Ongole grade cattle (Figure 2) and 7 SNPs with 6 haplotypes in Brahman cattle (Figure 3). Four samples of fifteen Brahman samples had SNP which located at 3 different places from the SNP on beef PO Kebumen. There were 2 samples (CB9/H-VI and CB12/H-V) with nucleotide changes from Guanine to Adenine, then on the two and three positions respectively (CB15/H-IV and CB7/H-III) Cytocin changed into Adenine and Guanine changed into Adenine (Figure 4, on the last 4 lines). The 11 other samples could be divided into two groups of haplotype, namely H-I (CB1, CB2, CB3, CB10, CB11, and CB14) and H-II (CB4, CB5, CB6, CB8, and CB8). While 4 SNPs of 7 SNPs on 11 samples of the Brahman cattle were located at the same locus of the 4 SNPs of Kebumen Ongole grade cattle. So, the 4 SNPs included in the 36 SNPs in Kebumen Ongole grade cattle were identified.

Sample	Single Nucleotide Polymorphism (1-7)							H
	1	2	3	4	5	6	7	
CB 1	2	1	3	3	4	2	4	I
CB 2	2	1	3	3	4	2	4	I
CB 3	2	1	3	3	4	2	4	I
CB 5	2	1	3	3	4	2	4	I
CB 10	2	1	3	3	4	2	4	I
CB 11	2	1	3	3	4	2	4	I
CB 14	2	1	3	3	4	2	4	I
CB 4	2	2	4	3	3	2	2	II
CB 6	2	2	4	3	3	2	2	II
CB 8	2	2	4	3	3	2	2	II
CB 13	2	2	4	3	3	2	2	II
CB 7	2	1	3	3	4	1	4	III
CB 15	2	1	3	1	4	2	4	IV
CB 12	1	1	3	3	4	2	4	V
CB 9	1	2	4	3	3	2	2	VI

Figure 3. Identification of haplotype based on Cyt b gene in Brahman cattle

**Comparative Haplotype Analysis between Kebumen Ongole Grade Cattle and Brahman Cattle**

Comparative analysis of haplotypes between Kebumen Ongole grade cattle and Brahman cattle can be seen in Figure 4. There were 8 haplotypes in Kebumen Ongole grade cattle and 6 haplotypes in Brahman cattle in this study. Haplotype V in Kebumen Ongole grade cattle was similar with haplotype I on Brahman cattle. It was presumed that haplotype V in Kebumen Ongole grade cattle came from Brahman cattle's dam. These results can be used as the basis of recommendations in germ selection to be developed as the dams. The percentage of haplotype for Kebumen Ongole grade cattle were successively haplotype I (14.29%), II (28.57%), III (7.14%), IV (7.14%), V (7.14%), VI (21.43%), VII (7.14%), and VIII (7.14%). While the results of the identification of haplotype for Brahman cattle were successively hap-

lotype I (46.67%), II (26.67%), III (6.67%), IV (6.67%), V (6.67%), and VI (6.67%).

**Results of Linkage Disequilibrium (LD) Analysis**

The results of the LD analysis for Brahman cattle were presented in Figure 5. The results showed that there were 21 SNP variation pairs on Brahman cattle. From the 21 SNP variation pairs, 28.57% of SNPs had high values for LD (> 8) and 71.43% having low LD values. The pairs with high LD values in Figure 6 are marked with black (D' and R2 value= 1). It means that the relationships between SNPs have a maximum value (100%). The low LD values were marked with white and gray. Based on the result, there were 4 kinds of SNP that were recommended to be used i.e., SNP 2, 3, 5, and 7. SNP 1, 4, and 6 were not recommended because of each SNP that paired with another SNP generated the low values of D' and R.

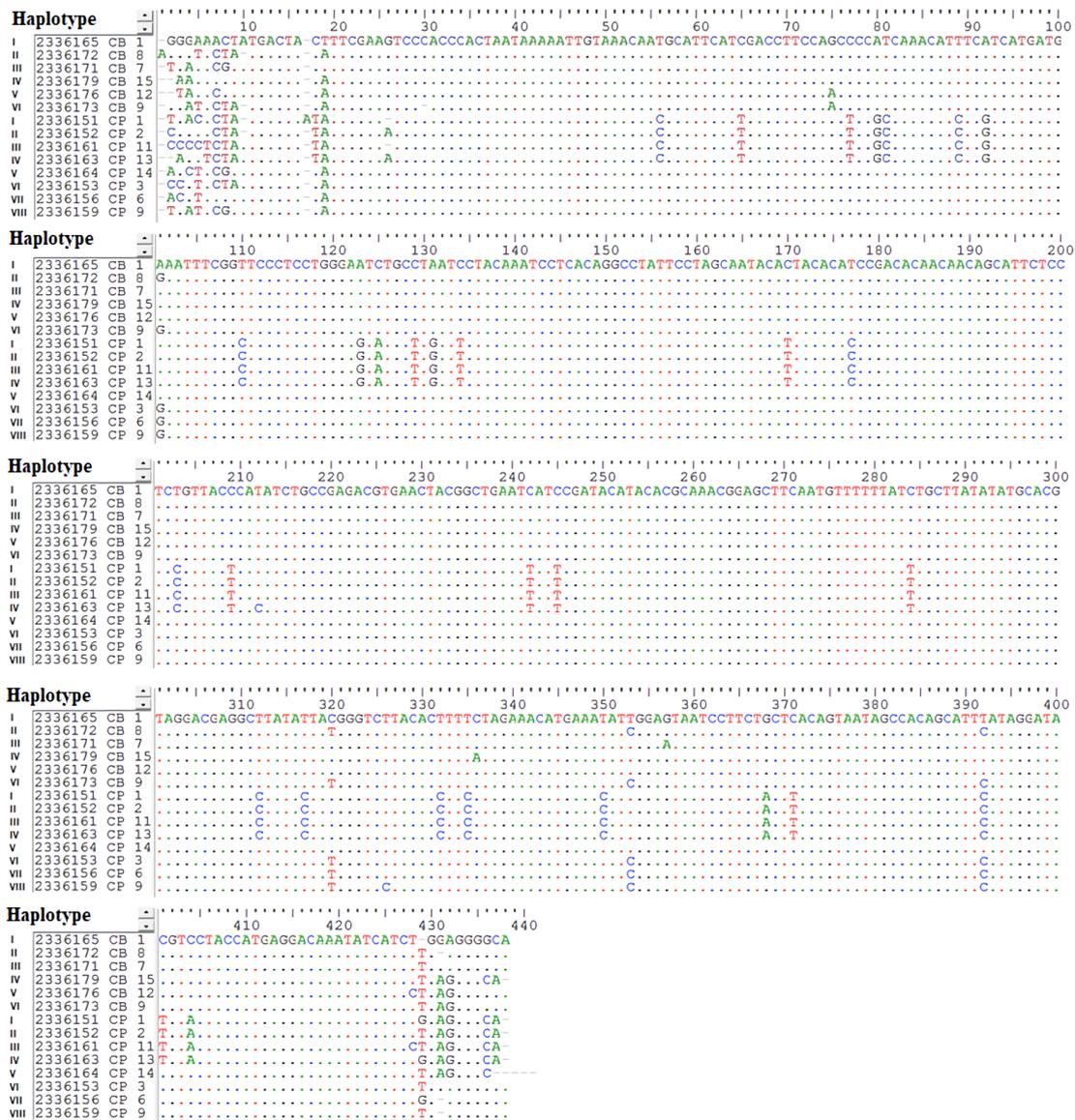


Figure 4. The haplotypes of cyt b sequence in Kebumen Ongole grade cattle and Brahman cattle

The result of LD analysis on Kebumen Ongole grade cattle using 34 SNP variations generated a total of 562 pairs SNP. A total of 72.37% SNP had high values of LD. There were three pairs of SNP having high value of  $R^2$  but their LD values were low (SNP 7-24, 7-29, 24-29), so they were also not recommended. SNP 7, 18, 24, 25, and 29 in Kebumen Ongole grade cattle were not recommended because of producing the low values of LD and R. The results of the LD analysis for Kebumen Ongole grade cattle were presented in Figure 6. The illustration at Figure 6 shows the visible shades of gray with numbers 74 indicating the variation values of D (>80%), it means they are of reasonable strength but not maximum. While the white color is the haplotype pairs with the low values of  $R^2$  and  $D'$ .

DISCUSSION

Mitochondrial DNA has been commonly used as genetic markers for identification and characterization because it is stronger than nuclear DNA (Chung, 2013). Identified target of single nucleotide polymorphism in the mtDNA Cyt-b gene was derived from multiple alignments of Gen-Bank sequences (accession No. AF492350, AY126697, JN817304, JN817303, JN817330, JN81730, and GU256940). The results of mtDNA Cyt b gene alignment showed five types of haplotype (data were not shown). Haplotype was the combinations of alleles which present on each of the two homologous chromosomes in a diploid individual (Miar *et al.* 2016). Hartatik *et al.* (2014) reported that there were only two haplotypes (A and B) found in Indonesian local cattle. The higher total number of haplotype was shown by Chung *et al.* (2013) in Korean Native Cattle and Japanese Black cattle i.e., 10 and 14, respectively. Difference of the number haplotype could be due to the differences of the samples used in the study.

In the present study, sequencing analysis on mtDNA cyt-b gene showed 36 SNPs with 8 haplotypes within Kebumen Ongole grade cattle (Figure 2) and 7 SNPs with 6 haplotypes within Brahman cattle. Chung (2013) reported a lower number of SNP in Korean Native Cattle (KNC) i.e., 29 SNPs between cattle populations. The SNP number in cyt b genes could be used for predicting genetic diversity (Cai *et al.*, 2007).

There were 29 SNPs out of 36 SNPs in Kebumen Ongole grade cattle which were different from the SNP in Brahman cattle. The other 7 SNPs out of 36 SNPs in Kebumen Ongole grade cattle had the same variation on both types of cattle. One of the Kebumen Ongole grade cattle samples had 100% similarities to Brahman cattle, which meant that Kebumen Ongole grade cattle had the

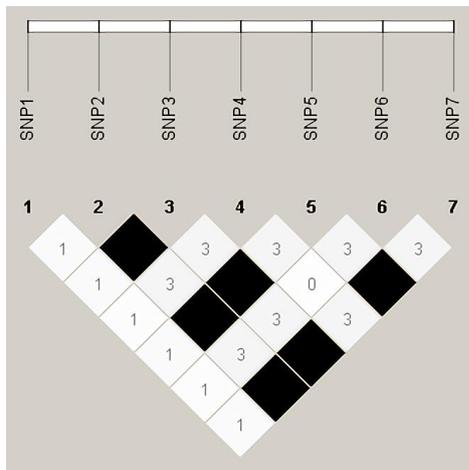


Figure 5. The plot of linkage disequilibrium (LD) of the cyt b gene SNPs in Brahman Cattle estimated using the haploview software

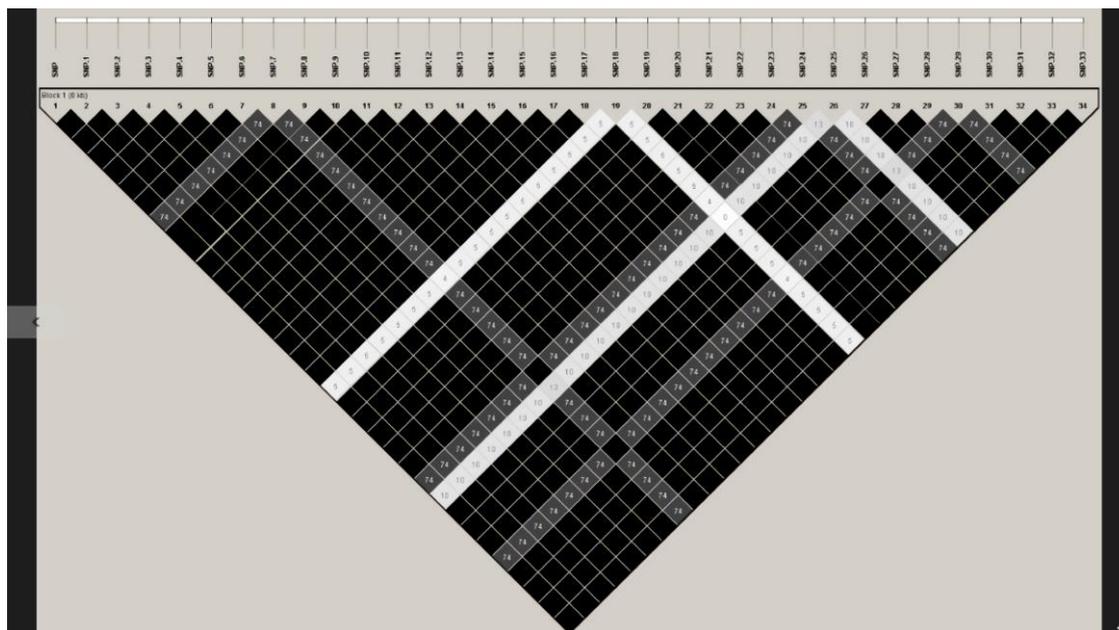


Figure 6. The plot of linkage disequilibrium (LD) of the cyt b gene SNPs in Kebumen Ongole grade cattle estimated using the haploview software

same nucleotide with Brahman cattle. This result probably indicated that Kebumen Ongole grade cattle which have the same sequence with Brahman cattle have the same origin of maternal inheritance. Thus, these SNP could be used to detect the purity of Kebumen Ongole grade cattle. Chung (2013) reported that the whole sequences of mtDNA could be used to understand the relationship between two different breeds of cattle. Kebumen Ongole grade cattle with haplotype V (CP14) had 100% similarities with haplotype I (CB 1, 2, 3, 10, 11) on Brahman cattle (Figure 3-4). These results strengthen the argument that there is a relationship between them. The other results also showed that 50% of Kebumen Ongole grade cattle (7 of 14 samples) had the same mtDNA sequence with Brahman cattle i.e., 85% identical. Sutarno & Setyawan (2015) reported that in 2006, Brahman cattle was massively distributed throughout Indonesia to support the government program. Artificial insemination by Brahman semen was more interesting because of fast-growing livestock product.

LD also useful for marker assisted selection (MAS), quantitative trait loci (QTL) mapping, parentage testing, and fine mapping of genetic diseases (Espigolan *et al.*, 2013). The value of LD will be higher when crossing inbred lines and lower when crossing breeds undifferentiated in gene frequencies (Jasielczuk *et al.*, 2016). We present LD statistics in the diagram based on  $r^2$  values using the haploview software. Measuring the LD,  $D'$ , and  $r^2$  were used to predict LD value between markers (Gurgul *et al.*, 2014).  $R^2$  parameter had better interpretable measure of LD (Qanbari *et al.*, 2010). The influence of allele frequency against  $r^2$  also lower compared to  $D'$  in a limited population (Mokry *et al.*, 2014).

The results of the LD analysis for Brahman cattle showed 21 SNP variations and 28.57% of SNPs had high values for LD ( $D' > 8$ ) and 71.43% having low LD values. The result of LD analysis in Kebumen Ongole grade cattle generated a total of 562 SNP pairs and 72.37% SNP having high values of LD. The pairs with high LD values marked in black ( $D'$  and  $R^2$  value =1) and the low LD values were marked in white and gray. The previous LD analysis study in Limousin, Simmental, and Polish Red cattle had generated a higher SNP panel, namely, 40,158, 40,117, and 42,118, respectively (Jasielczuk *et al.*, 2016). A group of 4,525 SNPs were observed and used for LD analysis in Hanwoo cattle (Lee *et al.*, 2011). The different in the number of SNP pairs could be caused by the cattle breeds and the number of samples used in these studies.

In this study, 28.57% SNP of Brahman and 72.37% SNP of Kebumen cattle had high values of LD ( $D'$  and  $R^2$  value =1). This result was higher than LD which observed in Limousin, Simmental, and Polish Red cattle which were 0.27, 0.01, and 0.02, respectively (Jasielczuk *et al.*, 2016). Other studies of LD in eight breeds of cattle (Angus, Charolais, Dutch Black, White Dairy, Holstein, Limousin, Nelore, Brahman, and Japanese Black cattle) were shown to be not more than 0.5 (McKay *et al.*, 2007). We recommended these SNP which had the higher value of LD to be used for the analysis of selection intensity and general level of genetic diversity.

## CONCLUSION

Kebumen Ongole grade and Brahman cattle had blood relations based on mtDNA Cyt b. The number of SNP and the value of LD on Kebumen Ongole grade cattle was greater than Brahman cattle. Therefore, the information on SNP and haplotype can be used as a marker to differentiate between Kebumen cattle and Brahman cattle.

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