

## Short Communication:

# Genetic diversity and phylogenetic analysis of two Indonesian local cattle breeds based on cytochrome *b* gene sequences

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**Abstract.** Hartatik T, Hariyono DNH, Adinata Y. 2019. Genetic diversity and phylogenetic analysis of two Indonesian local cattle breeds based on cytochrome *b* gene sequences. *Biodiversitas* 20: 17-22. Genetic diversity and phylogenetic relationships of two Indonesian local cattle breeds (Pasundan and Pacitan cattle) were investigated using mitochondrial DNA (mtDNA) cytochrome *b* (cyt *b*) gene analysis. Partial sequences of cyt *b* gene, 404 bp in length, were determined for 21 individuals from the two breeds. Genetic diversity of the breeds was assessed based on the number of polymorphic sites, number of haplotypes, haplotype diversity, nucleotide diversity and average number of differences. In addition, a neighbour-joining (NJ) haplotype tree was constructed based on Kimura's two-parameter model. Among the two breeds, haplotype and nucleotide diversity of Pacitan cattle were the highest with values of 0.3778 and 0.00099, respectively. In contrast, Pasundan cattle had the lowest value for haplotype (0.1818) and nucleotide (0.00045) diversity. Four haplotypes (Hap\_16, Hap\_17, Hap\_18 and Hap\_19) were found across the two breeds and around 85.71% of investigated individuals were classified as Hap\_16. Phylogenetic analysis with the inclusion of the cyt *b* sequences from 39 cattle breeds from Genbank database, showed that Indonesian cattle made a separated lineage together with *Bos javanicus*, *B. bison*, and *B. bonasus*. Pasundan and Pacitan cattle were considered from the same lineage based on haplotype distribution as well as phylogenetic analysis. This study may help the future researchers and livestock breeders for designing a breeding program based on a better understanding of the genetic diversity and history of local breeds.

**Keywords:** Cytochrome *b*, Genetic diversity, Indonesian cattle, mtDNA, phylogenetic analysis

## INTRODUCTION

During its uncertain history, a number of local cattle breeds have contributed to the Indonesian livestock industry to date. Mostly, Indonesian local cattle breeds are descendants of the Indian zebu (*Bos indicus*) and banteng (*B. javanicus*) (Mohamad et al. 2009). As a part of the local cattle breeds in Indonesia, Pasundan and Pacitan cattle are known for being well adapted to harsh environmental conditions with fluctuation in feed quality. Pasundan cattle (*B. javanicus* x *B. indicus*) are commonly found in the province of West Java particularly in Ciamis (Sutarno and Setyawan 2015). Whereas, Pacitan cattle are bred across Pacitan of East Java. Their role is clear, they provide a source of immediate income and food for households.

To date, for the sustainable use of livestock breeds, there are major barriers and mostly, these are probably due to lack of information regarding the existing local breeds or populations, geographical locations and genetic characteristics (Long 2008). In addition, current proper strategies for livestock utilization mainly depend on the deep understanding of genetic variation, which exists within and between breeds. In both Pasundan and Pacitan cattle, no molecular information is available so far. A considerable effort has focused on molecular characterization in major cattle breeds in Indonesia, such as

Bali (Winaya et al. 2009; Hartatik et al. 2015; Septian et al. 2015; Sutarno et al. 2015) and Madura cattle (Hartatik et al. 2013).

With the advanced growth of molecular techniques, employment of molecular markers will be the best approach to understand the existing genetic variation of the analyzed local breeds. Mitochondrial DNA (mtDNA) represents only a tiny fraction of organism genome size, yet it has been by far the most popular marker of molecular diversity in animals over the last three decades (Galtier et al. 2009). From a populational perspective, it could be considered as a system of small, sexually isolated demes, or clonal lineages, with an evolutionary rate 5 to 10 times faster than the nuclear genome, and such characteristics, therefore, make this molecule ideal for evolutionary studies (Castro et al. 1998). In mtDNA, the cytochrome *b* (cyt *b*) gene contains abundant phylogenetic information among intra- and interspecies and it is considered to be a good marker to study the genetic differentiation as well as phylogenetic relationships among species within the same genus or the same family (Browsers et al. 1994; Zardoya and Meyer 1996). The mtDNA has been proven to be useful markers for elucidating genetic diversity and relationships of domestic animals including chicken, goat, pig, horse, and cattle (Zhu et al. 2014; Sephri and

Syedabadi 2015; Hartatik et al. 2016; Sziszkosz et al. 2016; Hartatik et al. 2018).

To date, there has been no literature on genetic characterization of Pasundan and Pacitan cattle. Focusing on the genetic diversity of the locally adapted breeds, in this study, we attempted to gain more understanding about genetic diversity and phylogenetic relationship of Pasundan and Pacitan cattle breeds using a partial sequence of mtDNA *cyt b*. This study should have implications for more effective management and utilization of this valuable genetic resources.

## MATERIALS AND METHODS

### Sample collection and DNA extraction

Blood samples were randomly collected from 21 individuals from Pasundan and Pacitan cattle (Table 1), then stored at -20°C in 3 ml EDTA vacutainer tube. DNA extraction was performed using gSYNCTM DNA extraction kit (Geneaid, New Taipei City, Taiwan). The extracted DNA were tested with agarose gel electrophoresis and stored at -20°C before further analysis.

### PCR amplification and sequencing

To amplify the 464 bp of the partial mtDNA *cyt b*, a forward primer L14735 (5'-AAA AAC CAC CGT TGT TAT TCA ACT-3') and a reverse primer H15149 (5'-GCC CCT CAG AAT GAT ATT TGT CCT CA- 3') were used (Wolf et al. 1999). The PCR reaction was carried out in a final volume of 20 µl containing 1 µl genomic DNA (10-100 ng), 1 µl each primer, 10 µl PCR KIT (KAPPA2GTM Fast, KAPABIOSYSTEMS, USA) and 7 µl ddH<sub>2</sub>O. PCR amplification was carried out at a pre-denaturation step of 2 min at 94°C, 35 cycles of 36 sec at 95°C, 73 sec at 51°C, and 84 sec at 72°C, followed by a final extension step of 3 min at 72°C using Primus-25 Advanced Thermal Cycler. The PCR products were then visualized on 1% agarose gel electrophoresis and sequenced using Genetic Analyzer 3730xl (Applied Biosystems, Foster City, CA, USA).

### Data analysis

Firstly, the 464 base pairs (bp) PCR-amplified fragment of the *cyt-b* region of the 21 individuals were successfully sequenced. All sequences were edited manually using BioEdit package and were aligned with 39 available sequences (Genbank database) of *cyt b* from other cattle breeds using CLUSTAL W (Thompson et al. 1994). The available sequences of *cyt b* included Asian cattle (GenBank Accession No. AY526085, DQ124389, AY885283 and DQ186203), American cattle (GenBank Accession No. AY676860, AY676861 and AY676866), European cattle (GenBank Accession No. V00654, AF492351, EU177834, DQ124413, GQ129208, EU177847, EU177852, EU177862, EU177867, EF693798, EU747736 and GQ129207), *B. indicus* (GenBank Accession No. AF492350, AF492351, AF531473, AY126697 and AY689190), *B. grunniens* (GenBank Accession No. AF091631, AY684273, AY955225, EF494177, EF494178, EF494179 and NC\_006380), *B.*

*javanicus* (GenBank Accession No. AY689188, EF197952, D34636, D82889, DQ459558, DQ459559), *B. bonasus* (Y15005) and *B. bison* (AF036273). As a result, 404 bp of *cyt b* gene in length were determined based on their overlapping sites and the quality of sequencing peak for individuals (Figure 1). Genetic diversity indices including the number of polymorphic sites, number of haplotypes, haplotype diversity, nucleotide diversity and average number of differences were performed in DnaSP ver. 6.10.04 (Rozas et al. 2017). MEGA ver.7.0 (Kumar et al. 2016) was used to construct neighbor-joining (NJ) tree with Kimura 2-parameter model (bootstrap value is 1000).

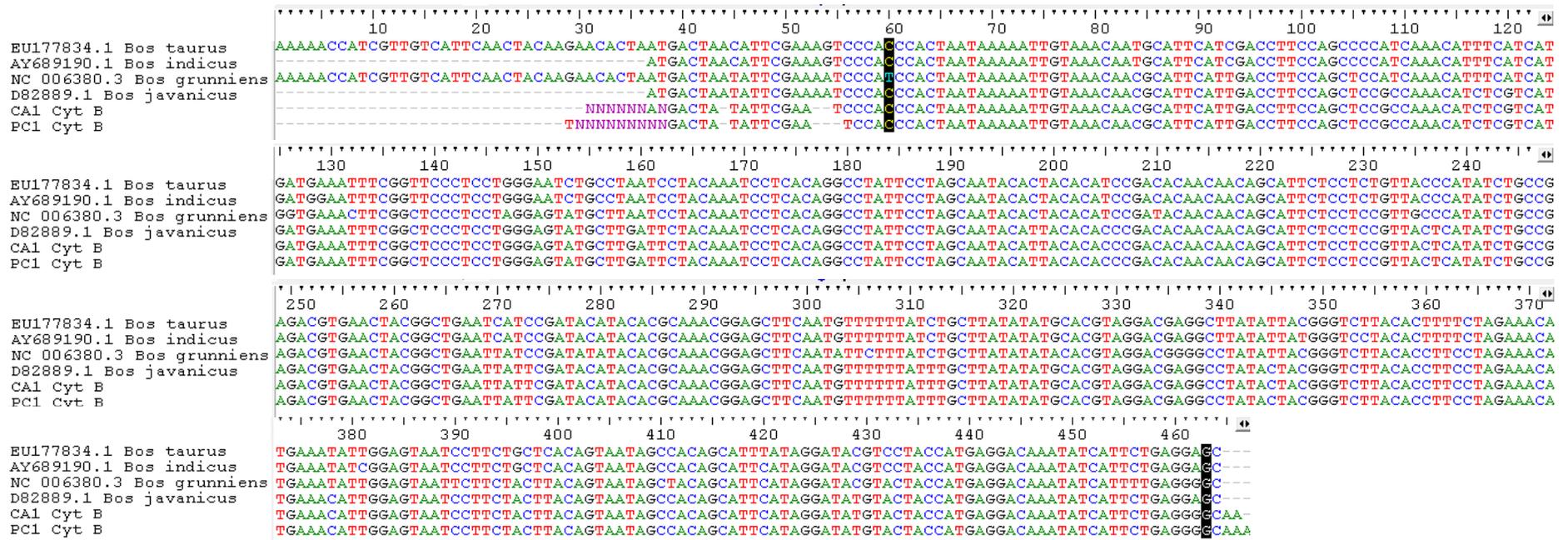
## RESULTS AND DISCUSSION

### Sequence variation and genetic diversity

The 404 bp of *cyt b* sequences from 21 individuals of Indonesian local cattle were analyzed. Among the two investigated breeds, the compositional frequency of A base was the highest (30.2%) among three other bases (T, 27.2%; C, 26.00%; G, 16.3%). The nucleotide composition in this study was similar to published studies in cattle (Kim et al. 2013), sheep (Pakpahan et al. 2016; Sofla et al. 2017) and chicken (Adamu et al. 2016), suggesting that composition of A+T nucleotides in those animals was higher than C+G nucleotides.

Genetic diversity indices among the two investigated breeds are presented in Table 1. In this study, one and two polymorphic sites were identified in all sequences of Pasundan and Pacitan cattle, respectively. Nucleotide diversity ranged from 0.00045 to 0.00099 with an average number of differences of 0.182 in Pasundan cattle and 0.400 in Pacitan cattle. Low genetic diversity was observed in the two breeds, depicted by the low value of haplotype diversity, 0.1818 for Pasundan cattle and 0.3778 for Pacitan cattle, which were lower than that observed in Korean cattle (Kim et al. 2013). On the other hand, Sutarno et al. (2015) reported the moderate magnitude of genetic diversity of Indonesian local cattle breeds. The possible reasons for low haplotype diversity in both populations would be a limited number of sequences, restricted area of the breeding region, as well as inbreeding.

Pasundan and Pacitan cattle are breeds, mostly reared in the west and east provinces of Java island, respectively. Despite the important role of local cattle as a food source and a valuable genetic resource, several populations have been suffering a drastic reduction in the last few years because of uncontrolled mating. Moreover, several cases such as inbreeding, genetic problems, and diseases had threatened the farm animal genetic resources. As noted by Sutarno (2015), two threats to the sustainability of livestock are inbreeding and loss of genetic variation. Based on the current findings, the careful utilization of the studied populations based on genetic criteria can provide a greater chance for more effective management. Also, preventing further loss of genetic diversity is crucial for the populations which had low diversity.



**Figure 1.** Partial sequences of *cyt b* gene. The sequence' beginning and ending points are indicated by letters highlighted in black (404 bp in length) used for further analysis

**Table 1.** Genetic diversity indices in Indonesian local cattle breeds

Breed	Number of individuals	Number of polymorphic site	Number of haplotype	Haplotype diversity	Nucleotide diversity	Average number of differences
Pasundan	11	1	2	0.1818	0.00045	0.182
Pacitan	10	2	3	0.3778	0.00099	0.400

**Table 2.** Haplotype distribution *cyt b* gene sequences in investigated cattle breeds with Genbank database

Breed	Sample/GenBank accession number	Haplotype	Breed	Sample/GenBank accession number	Haplotype
Pasundan (present study)	CA1	Hap_16	European	EU177834	Hap_1
	CA2	Hap_16		DQ124413	Hap_4
	CA3	Hap_16		GQ129208	Hap_1
	CA4	Hap_16		EU177847	Hap_1
	CA5	Hap_16		EU177852	Hap_1
	CA6	Hap_16		EU177862	Hap_1
	CA7	Hap_16		EU177867	Hap_1
	CA8	Hap_16		EF693798	Hap_1
	CA9	Hap_16		EU747736	Hap_1
	CA10	Hap_16		GQ129207	Hap_1
	CA11	Hap_17	<i>B. indicus</i>	AF492350	Hap_5
Pacitan (present study)	PC1	Hap_16	<i>B. grunniens</i>	AF531473	Hap_5
	PC2	Hap_16		AY126697	Hap_5
	PC3	Hap_16		AY689190	Hap_6
	PC4	Hap_18		AF091631	Hap_7
	PC5	Hap_19		AY684273	Hap_8
	PC6	Hap_16		AY955225	Hap_9
	PC7	Hap_16		EF494177	Hap_10
	PC8	Hap_16		EF494178	Hap_10
	PC9	Hap_16		EF494179	Hap_7
	PC10	Hap_16		NC_006380	Hap_7
Asian	AY526085	Hap_1	<i>B. javanicus</i>	AY689188	Hap_11
	DQ124389	Hap_2		EF197952	Hap_12
	AY885283	Hap_1		D34636	Hap_11
	DQ186203	Hap_3		D82889	Hap_11
American	AY676860	Hap_1		DQ459558	Hap_13
	AY676861	Hap_1		DQ459559	Hap_12
European	AY676866	Hap_4	<i>B. bonasus</i>	Y15005	Hap_14
	V00654	Hap_1	<i>B. bison</i>	AF036273	Hap_15
	AF492351	Hap_1			

As shown in Table 2, a total of 39 reference sequences of *cyt b* from other cattle breeds (Asian, American, European, *B. indicus*, *B. javanicus*, *B. grunniens*, *B. bonasus* and *B. bison*) were included for the haplotype distribution analysis. As a result, nineteen haplotypes (Hap\_1 to Hap\_19) were identified across all analyzed sequences of *cyt b*. There were 2 haplotypes in Pasundan cattle and 3 haplotypes in Pacitan cattle. Hap\_16 was the most common haplotype among the two breeds. There were no other breeds included in this haplotype. The Hap\_16 evidenced that both populations can be attributed to same maternal origins. Also, Hap\_16 suggested that a

low level of genetic diversity was present within and between these breeds.

Interestingly, with a few samples observed in this study, we also detected specific haplotypes including Hap\_17 (in Pasundan cattle), Hap\_18 and Hap\_19 (in Pacitan cattle), with only one individual of each haplotype (Table 2). Specific haplotypes were also found in Aceh cattle inferred from D-loop sequences (Sari et al. 2016). In addition, Adinata et al. (2017) identified specific haplotypes in Madura cattle inferred from *cyt b* sequences. Since the investigated Indonesian cattle are important genetic resources, these observed specific haplotypes, therefore,

should be maintained and preserved.

### Genetic distance of Indonesian, Asian, American, European cattle and *Bos javanicus*

Estimate of genetic distance based on the model of parameter Kimura was performed to analyze the genetic distances of Indonesian cattle and other cattle breeds (Asian and European cattle as well as *B. javanicus*), which were obtained from genbank database. The results showed that the genetic distance between Indonesian cattle and other cattle breeds ranged from 0.002 to 0.077. The estimated genetic distances was the closest between Indonesian cattle and *B. javanicus* with a value of 0.002. This is due to the fact that Indonesian cattle were derived from banteng (*B. javanicus*) and zebu (*B. indicus*). Bali cattle, an indigenous cattle in Indonesia, is the only domesticated banteng exists in this country. The current findings provided an evidence that *B. javanicus* blood in the investigated cattle breeds was possibly from Bali cattle. Meanwhile, the analysis of mitochondrial DNA reported by Nijman et al. (2003) showed that in Indonesian cattle of Madura and Bali-Malaysia cattle, the allele from zebu cattle was found. In addition, a close genetic distance between Indonesian cattle of Aceh and zebu cattle was observed as reported by Sari et al. (2016).

### Phylogenetic analysis

In this study, a neighbor-joining (NJ) tree was generated in order to verify the phylogenetic relationship of the 19 haplotypes of the *cyt b* gene using MEGA software ver. (7.0.14) with bootstrap replications of 100% (Figure 2). The results explained that the Indonesian local cattle breeds (Pasundan and Pacitan cattle), which belonged to Hap\_16, Hap\_17, Hap\_18 and Hap\_19 were verified to have mtDNA maternal lineages from *B. javanicus* (Hap\_11, Hap\_12 and Hap\_13). Also, the constructed tree demonstrated that our studied populations were clearly separated from Asian, American and European cattle lineages. As previously reported by Mohamad et al. (2009), the *B. javanicus* introgression of 10–16% (mitochondrial) and 20–30% (Y-chromosomal) has been indentified in Indonesian zebu breeds (Madura and Galekan cattle), whereas no evidence for zebu introgression has been observed in five populations of the Bali cattle, a domestic form of the banteng. Other Indonesian cattle breeds (Madura and Java cattle) were closely related to *B. javanicus*, as reported by Hartatik et al. (2015).

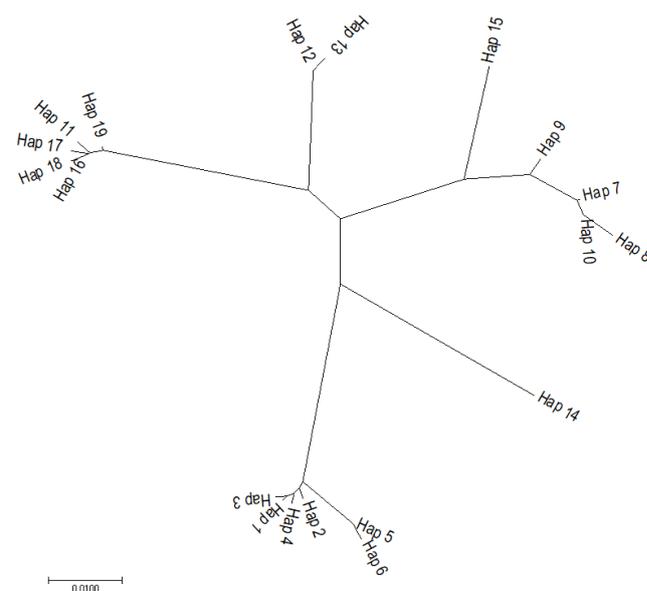
Interestingly, our findings highlighted that the Pasundan and Pacitan cattle, representing four haplotypes, shared only one common maternal ancestor in the same lineage. It was noted that Hap\_16 was quite frequently shared between Pasundan dan Pacitan cattle, indicating a close genetic relationship between these two studied breeds. According to Nijman et al. (2003), hybridization between breeds may occur if they shared an overlapping habitat or as a result of human interference during captive. However, in reality, the breeding region of the two populations were far away one to another. The possible reason for the close relationship between them is the genetic introgression of closely related maternal genes from *B. javanicus*. Based on

the estimated genetic distances (Table 3), the Indonesian local cattle were genetically closer to *B. javanicus* (0.002) than to Asian (0.074), American (0.077) and European (0.074) cattle. This result supported the findings of Mohamad et al. (2009), indicating that Indonesian local cattle were derived from *B. javanicus* and *B. indicus*.

Finally, this study has provided information regarding the genetic diversity of Indonesian local cattle breeds (Pasundan and Pacitan cattle). Also, we performed a phylogenetic analysis of them together with other cattle breeds. Four haplotypes and relatively low genetic diversity were found in the present breeds studied. Indonesian cattle breeds were distantly related to other Asian, American, and European cattle breeds, which suggests that both Pasundan and Pacitan cattle may have a unique gene pool compared to these mentioned breeds. The finding of this study is quite significant for better management and conservation of Indonesian cattle breeds since very little information is available about Pasundan and Pacitan cattle phylogenetic as well as their genetic diversity.

**Table 3.** Estimates of genetic distance among cattle breeds

	[1]	[2]	[3]	[4]	[5]
[1] Asian cattle	-				
[2] American cattle	0.002	-			
[3] European cattle	0.005	0.002	-		
[4] <i>B. javanicus</i>	0.072	0.074	0.072	-	
[5] Indonesian cattle	0.074	0.077	0.074	0.002	-



**Figure 2.** Phylogenetic relationship among 19 haplotypes of the *cyt b* gene from Indonesian and other cattle breeds obtained from Genbank database by neighbor-joining (NJ) method.

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