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Polymorphism of Cyt-b Gene in Several Indonesian Cattle Using PCR-RFLP Method

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ABSTRACT

The cytochrome b (Cyt-b) gene is one of the genes that is located in the mitochondrial DNA. Variations in the Cyt-b gene can be used to compare different animal species to investigate the origin of certain animal species. This study aimed to assess the genetic diversity of Indonesian local cattle breeds, including Bali cattle as an Indonesian native cattle breed and Banteng as the wild type of Bali cattle, using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). A total of 336 individual DNA samples from Indonesian cattle breeds were analyzed in this study. The RFLP method using three restriction enzymes, i.e., HinfI, HaeIII, or XbaI, was used to identify the variation of the Cyt-b gene. The Cyt-b gene was polymorphic based on the PCR-RFLP method. There were six alleles of the Cyt-b gene found in this study, i.e., A and B allele (HinfI), C and D allele (HaeIII), X and Y allele (XbaI). All alleles can be found in Pasundan, Madura, and PO cattle. Pesisir cattle have the highest allele frequency D. The UPGMA results showed three clusters of Indonesian native cattle in this study. Cluster 1 consists of Pasundan, Banteng, and Bali cattle. Cluster 2 consists of Madura, PO, and SO cattle. Pesisir was separated from other cattle in cluster 3. The X allele could become an indicator to distinguish Banteng and Bali cattle.

Keywords: Cytochrome, Local cattle, Mitochondria, PCR-RFLP, Polymorphism

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Introduction

Indonesia has Bali cattle as native cattle (Hardjosubroto, 1994) and many local breeds of cattle, including Aceh, Pesisir, Grati, Jabres, Pasundan, Galekan, Sumba Ongole, Ongole Grade, Madura, Katingan, Sumbawa, and Donggala (the characteristics were described in Sutarno and Setyawan (2015)). A well-designed breeding program for Indonesian cattle breeds was needed to improve their socio-economic value. To perform a conservation program, especially regarding Indonesian cattle resources, information about the genetic diversity of certain cattle breeds is needed, and it would be a helpful tool for farmers to develop their businesses. In addition, determining the number of the initial (base) population to produce offspring consistent with the objectives of the breeding program is a key factor for the success of the breeding program (Agung *et al.*, 2015).

Mitochondria have been well-known as the powerhouses of the cell due to their most primary function being oxidative phosphorylation (Ladoukakis and Zouros, 2017). Mitochondrial DNA is extensively used to define genetic similarity based on the maternal line. In addition, genetic diversity and genetic structure in certain animal breeds can be investigated using mitochondrial DNA (Sharma *et al.*, 2015). Mammalian mitochondrial DNA encodes 11 messenger RNAs (mRNAs) (translated to 13 proteins), 22 tRNAs, and 2 ribosomal RNAs (rRNAs) (12S and 16S rRNA) (Gustafsson *et al.*, 2016). One gene in the mitochondrial DNA is the cytochrome-b (Cyt-b) gene (Stewart and Chinnery, 2015). Information about polymorphism of the Cyt-b gene can be useful for investigating the origin of certain animal species (Farag *et al.*, 2015) and also for a comparison study of different animal species (Munira *et al.*, 2016). This study aimed to assess the genetic diversity of Indonesian local cattle

breeds using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

Materials and Methods

Sample and DNA collection

A total of 336 individual DNA samples from Indonesian cattle breeds were analysed in this study including Pasundan (n=123; from West Java) Madura (n=18; from West Java) SO (n=36; from East Nusa Tenggara) PO (n=120; from East Java) Pesisir (n=13; from West Sumatera) Bali (n=12; from Nusa Penida, Bali) and Banteng (n=14; from East Java). Amplification of the Cyt-b gene was conducted using a pair of primer i.e. forward: (5'-aaaaccaccgttgattcaacta-3') and (reverse: 5'-gccctcagaatgatattgtcctca-3') (Hartatik *et al.*, 2015). The PCR process used KAPA2G Robust Hot Start Ready Mix PCR Kit (Kapa Biosystems, Cape Town, South Africa), forward and reverse primers (200 ng/ μ L), DNA samples (5-50 ng/ μ L), and H₂O up to 25 μ L final volume. The PCR program was set as follows: denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 30 sec, and a final extension at 72°C for 5 min on Mastercycler® Gradient (Eppendorf, Hamburg, Germany). The amplicons that were generated by PCR proceeded to RFLP analysis. A total of 3 μ L PCR products, 1.4 μ L H₂O, and 1 unit (\pm 0,6 μ L) of restriction enzyme HinfI, HaeIII, or XbaI including its buffer (New England Biolabs, USA) were mixed and incubated at 37°C for 1 hour. Visualization of the RFLP products was conducted by electrophoresis process (2% agarose gel, SyBr® staining, and captured in the GBOX documentation System (Syngene, UK)).

The data was analyzed using POPGEN version 1:32 program (Yeh and Boyle, 1997) to generate allele frequency, Nei's gene diversity (h), gene flow (nm), and UPGMA. Principal Component Analysis (PCA) was performed using Orange 3.27 (Demšar *et al.*, 2013).

Results and Discussion

Using the sequences of the Cyt-b gene from *Bos taurus* (GenBank Acc. No. AF492351.1), we found that our primer will attach between 14,115 bp and 14,578 bp (base pair) with a total length of our gene target of 464 bp (Figure 1). In 2019, the genetic diversity for Indonesian native cattle breeds has been carried out using microsatellite markers (Agung *et al.*, 2019). In other studies, partial sequences of mitochondrial DNA were evaluated to investigate the genetic diversity of Pasundan cattle (Salimah *et al.*, 2022), Pesisir cattle (Putri *et al.*, 2019), Bali cattle (Hikmawaty *et al.*, 2020), Madura cattle (Wulandari *et al.*, 2019), and Ongole cattle (Mubarak *et al.*, 2019). There were limited reports for mitochondrial DNA variation based on RFLP methods in several Indonesian cattle breeds. In this study, we used the same three restriction enzymes

in our previous study in SO and PO cattle breed (Agung and Hermansyah, 2018).



Figure 1. The primer attachment position and restriction enzymes cutting sites in the Cytochrome-b gene (Acc.No. AF492351.1).

The size of bands that were cut using the three restriction enzymes can be seen in Table 1. The three restriction enzymes used produced two types of variations, each of which was symbolized sequentially from HinfI, HaeIII, and XbaI as follows: A, B, C, D, X, and Y. Previously, we have been reported these alleles in SO and PO cattle breed (Agung and Hermansyah, 2018). Visualisation of all allele of the Cyt-b gene is shown in Figure 2. The A and B (HinfI) alleles in this study were also same as reported in Hartatik *et al.* (2015). In this study, the A allele were not found in Bali cattle population. In contrast, the Bali cattle in Hartatik *et al.* (2015) were all have the A allele. This could be caused by differences in the samples used. In this study, Bali cattle were collected from Nusa Penida island (Bali Province) otherwise the Bali cattle that reported in Hartatik *et al.* (2015) were from Kupang (East Nusa Tenggara Province).

Table 1. Cytochrome B fragment size is cut by the restriction enzyme

Restriction enzyme	Allele	Size
HinfI	A	198 bp, 149bp, 117 bp
	B	305 bp, 159 bp
HaeIII	C	179 bp, \pm 160 bp, \pm 120 bp
	D	285 bp, 179 bp
XbaI	X	344 bp, 100 bp
	Y	464 bp

Frequency of allele based on Cyt-b gene using RFLP can be found at Table 2. Pesisir cattle have the highest frequency of allele D compared to others. We found the highest gene diversity in XbaI (0.4995) (Table 3). On the other hand, the highest gene flow can be found in HaeIII loci. Based on the results, Banteng has only alleles B, Y, and C. Meanwhile, Bali cattle have alleles B, Y, C, and X. Bali cattle are considered a domesticated form of Banteng and become a native cattle breed in Indonesia (Hardjosubroto, 1994). So, allele X (XbaI) might be a differentiating indicator between Banteng and Bali cattle. Otherwise, all alleles were found in Pasundan, Madura, and PO cattle. In our previous study, the B (HinfI), D (HaeIII), and Y (XbaI) alleles were found only in the PO cattle, while the X (XbaI) allele was found only in the SO cattle (Agung and Hermansyah, 2018). However,

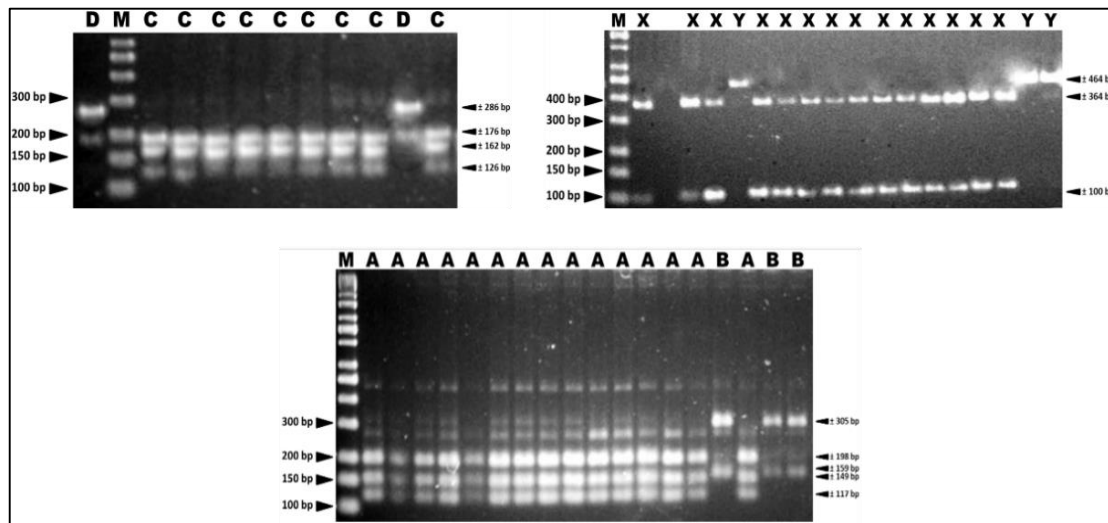


Figure 2. Alleles of the Cyt-b gene based on Agung and Hermansyah (2018).

Table 2. Frequency of allele

Breed	Allele					
	Hinfl		XbaI		HaeIII	
	A	B	X	Y	C	D
Pasundan (n=123)	0.0813	0.9187	0.0244	0.9756	0.9512	0.0488
Bali (n=12)	0	1	0.2	0.8	1	0
Madura (n=18)	0.2778	0.7222	0.8889	0.1111	0.7647	0.2353
SO (n=36)	1	0	0.9444	0.0556	1	0
PO (n=120)	0.1	0.9	0.8667	0.1333	0.9	0.1
Pesisir (n=13)	0.8333	0.1667	0	1	0.2308	0.7692
Banteng (n=14)	0	1	0	1	1	0
Overall (n=336)	0.216	0.784	0.4863	0.5137	0.9053	0.0947

n: number of samples.

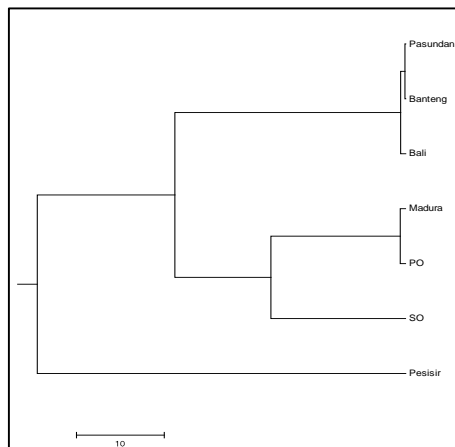


Figure 3. UPGMA result of several Indonesian local cattle breeds.

due to the opposite finding in this study it was no longer needed to proposed the B, D, X, and Y allele as a differentiation factor for SO and PO cattle breeds.

Table 3. Gene diversity and gene flow of the markers

Markers	h	nm
Hinfl	0.3387	0.243
XBA1	0.4996	0.1799
HaeIII	0.1714	0.526

h = Nei's gene diversity, nm = gene flow.

UPGMA results showed three clusters of Indonesian cattle in this study. Cluster 1 consists of Pasundan, Banteng, and Bali cattle. Cluster 2 consists of Madura, PO, and SO cattle (Figure 3). Pesisir was separated from other cattle in cluster 3. Based on HSP70 sequence, Madura cattle was genetically close to PO (Prihandini *et al.*, 2022). Based on principle component analysis, we found same result using UPGMA, Pesisir separated from others breed. Furthermore, Pasundan, Banteng and Bali in the same cluster (Figure 4). UPGMA and PCA generated same results. However, PCA

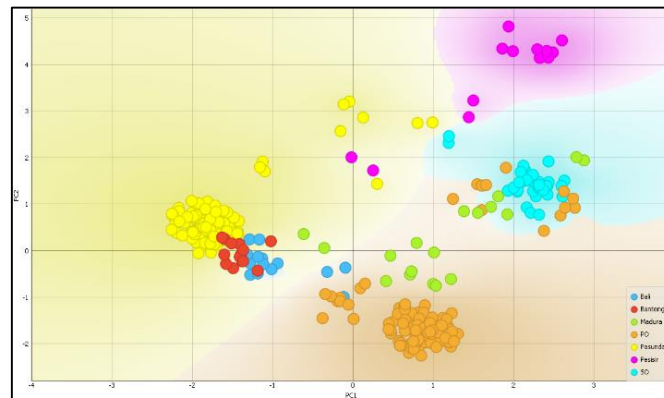


Figure 4. Principle component analysis of several Indonesian local cattle breeds based on Cyt-b gene.

can determine the distribution of data from existing alleles.

Conclusion

The Cyt-b gene were polymorphic in Indonesian cattle breeds based on PCR-RFLP method. The Cyt-b gene polymorphism was very high in Pasundan, Madura, and PO cattle breed. The X (XbaI) allele was very potential to become an indicator to distinguish Banteng and Bali cattle.

Conflict of interest

The authors of the manuscript have no conflict of interest to declare.

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Author's contribution

PPA, DP, AF, SS, and EMK carried out the study's design, data evaluation, and paper drafting. Sample preparation and analysis were carried out by FS and AH. KK, TES and MSAZ took part in the data collection process. The final manuscript was reviewed and approved by all authors.

Ethics Approval

This study was conducted under ethical approval of the National Research and Innovation Agency (BRIN), Indonesia (Register No. 077/KE.02/SK/10/2022).

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