BIODIVERSITAS
Volume 20, Number 9, September 2019
Pages: 2569-2575

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d200919

The origin of pesisir cattle based on *D-loop* mitochondrial DNA

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Manuscript received: 8 July 2019. Revision accepted: 20 August 2019.

Abstract. *Putri AE, Farajallah A, Perwitasari D. 2019. The origin of pesisir cattle based on* D-loop *mitochondrial DNA. Biodiversitas 20: 2569-2575.* Pesisir cattle is one of Indonesia's local cattle descended from *Bos indicus* which has small body characteristics. It was assumed due to crossbreeding between pesisir cattle and other cattle. Therefore this study aims to study the origin of pesisir cattle based on the *D-loop* mtDNA region and to find out the phylogenetic relationships between pesisir cattle and other cattle. Cattle blood samples were taken in two locations, Krui Way and Tanah Rekah. Amplification of the mitochondrial gene using the primary pair of collections of Dr. Achmad Farajallah, namely AF22 (forward) 5'-GCGTACGCAATCTTACGATCA-3' and AF23 (reverse) 5'-ATGCAGTTAAGTCCAGCTAC-3'. Reconstruction of phylogenetic trees was analyzed using the Neighbor-Joining Method based on Kimura 2 Parameters. The results of the mtDNA analysis in the *D-loop* region showed that there were two broods in pesisir cattle, namely *Bos indicus* and *Bos javanicus*. The closeness between these species is due to the close geographical distance between *Bos indicus* originating from Way Krui, Lampung and *Bos javanicus* from Java.

Keywords: *Bos indicus*, DNA, D-Loop, mitochondria, pesisir cattle

INTRODUCTION

Cattle are one of the domesticated livestock. The domestication of cattle started 8.000-10.000 year ago. It derived from auroch descendant (*Bos primigenius*) from Africa and distributed to Europa-Asia (Martinez-Navarro 2014). There are two types of modern cattle derived from *Bos primigenius;* those are humpless cattle (*Bos taurus)* and humped cattle (*Bos indicus*) (Troy 2001). Other than that, there is also *Bos javanicus*, which is descendant of *Bibos banteng*. *Bos javanicus* is one of the native Indonesian cattle, which is different from *B. taurus* and *B. indicus* (Nijman et al. 2003). One descendant of *B. javanicus* is an Indonesian Bali Cattle that distribute in all over Indonesia (Uggla 2008). There are also several types of domestic cattle in Indonesia, such as Aceh Cattle, Madura Cattle, and Pesisir Cattle (Martojo 2003).

Characteristics of pesisir cattle are unique. They have smaller body size than other cattle, short limb, slender legs and possessed small hump in the back. Pesisir cattle have monochrome color in body pattern that can be divided into red brick, yellow, brown, black, and white (Sarbaini 2004). West Sumatra society called pesisir cattle as Jawi Ratuih or Bantiang Ratuih, means cattle that breed once every year and have a lower price due to body size (Saladin 1983). Male pesisir cattle (age 4-6) have bodyweight around 160 kilograms, much lighter than that of Bali cattle (310 kilograms), PO cattle (388 kg), Aceh cattle (302 kg) and Madura cattle (248 kg) (Adrial 2010). The small weight may be caused by several causes, such as maintenance and feeding methods or crossbreeding between pesisir cattle and other cows.

Crossbreeding in pesisir cattle and other cows can occur because of the coexistence of the cattle in one area. The molecular method is one approach to study the crossbreeding. Various types of DNA markers can be used to understand the crossbreeding process. One of them is Mitochondrial DNA (Bradley 1996). Mitochondrial DNA was maternally inherited and showed a small amount of recombination, so it can be used to study the matrilineal genealogy of a species or between populations (Duryadi 1994). The most varied areas of mitochondrial DNA are the D-loop region (Pénzes et al. 2002). The *D-loop* region, which has a fairly high base variation, is located outside the segment that has a function of transcription and replication so it can be used to find out the pedigree of an animal and kinship (phylogenetic) (Mannen et al. 1998). Based on *D-loop*, Madura cattle showed two branches in phylogeny trees. Those are *B. indicus* and *B. javanicus* branch (Firdhausi 2010). Until now, there is no information about the phylogenetic relationship between Pesisir cattle (*B. indicus*) and other cattle using *D-loop* marker.

This study aimed to determine the origin of pesisir cattle based on mtDNA in *D-loop* region and evaluate the phylogenetic relationship between pesisir cattle and other cattle.

MATERIALS AND METHODS

Blood sample collection

This study was conducted in Way Krui Lampung and Tanah Rekah Bengkulu. The blood sample was taken using syringe by injecting it to the neck and tail parts. The volume of the blood sample was taken about 1.5-2 ml and was put in the vacutainer tube 15 ml. Then, it was preserved using alcohol 70% minimum two times the volume of the blood sample. There were 32 samples; those

consisted of 14 samples from Way Krui Lampung and 18 samples from Tanah Rekah Bengkulu (Table 1).

Total DNA isolation

The DNA was extracted using *Genomic DNA mini kit Geneaid*. This procedure followed the instruction from the *Genomic DNA mini kit Geneaid* (Geneaid Biotech Ltd., Taiwan)

mtDNA amplification in the D-loop region

The mitochondrial DNA amplification used a pair of primer from Dr. Achmad Farajallah collection, i.e., AF22 (forward) 5'-GCGTACGCAATCTTACGATCA-3' and AF23 (reverse) 5'-ATGCAGTTAAGTCCAGCTAC-3. The AF22 primer attaches to base 14979, and the primer AF23 attaches the base 16098 in Bos indicus (access number AF492350). In this amplification process, the size of the mtDNA that was targeted was 1120 bp. The pair of primers AF22 and AF23 located in the middle until the end of the DNA. This process used the PCR (*Polymerase Chain Reaction*) technique.

The composition of 25 ml PCR reaction was 1 ml (10-100 ng) of DNA sample, 1.25 unit of RBC Bioscience Taq polymerase and its buffer system, 1 ml dNTP ten nmol, 2 ml MgCl2, 1 ml primers AF22 and AF23, and DW sterile. All the compositions were mixed and then put in to the PCR tube. It was centrifuged in 300 rpm in 30 seconds. The result was amplificated in the Thermal Cycler TAKARA MP4 machine.

This procedure followed Firdhausi (2010) with some modification for the amplification process. We used 94^{o}C for 5 minutes for pre-denaturation, 94^{o}C for 1 minute for denaturation, 60^{o}C for 1 minute for annealing, and 64^{o}C for 1 minute for extension and was repeated for 30 cycles. The PCR reaction was ended with polymerization (final extension) at 72^{o}C for 2 minutes. The PCR product was visualized using electrophoresis gel technique with composition 6% polyacrylamide (PAGE) in 5xTBE (10 mM Tris-HCL, 1 M boric acid, 0.1 mM EDTA) buffer. The electrophoresis was run in 200 V for 45 minutes. The process then was continued with silver sensitive coloring (Tegelstrom 19869) with a little modification.

DNA sequencing

The amplification products that showed double bands were purified and being the templates in PCR reactions for nucleotide sequencing. The primers were used in the PCR process were the same as the primers were used for amplification. The PCR reaction was carried out using the Dideoxi Terminator method with labeled dNTP (big dye terminator). Nucleotide sequencing used sequencing services of First BASE Laboratories Sdn Bhd (Malaysia).

Table 1. Samples collection list

Sample number	Location	Gender	Ages
01	Way Krui	Female	Adult
03	Way Krui	Female	Adult
05	Way Krui	Female	Adult
07	Way Krui	Female	Juvenile
08	Way Krui	Female	Adult
09	Way Krui	Female	Adult
10	Way Krui	Female	Adult
11	Way Krui	Female	Adult
13	Way Krui	Female	Juvenile
14	Way Krui	Female	Adult
15	Way Krui	Female	Juvenile
16	Way Krui	Female	Adult
17	Way Krui	Female	Adult
18	Way Krui	Female	Adult
19	Way Krui	Female	Adult
21	Tanah Rekah	Female	Adult
22	Tanah Rekah	Female	Adult
23	Tanah Rekah	Male	Juvenile
25	Tanah Rekah	Female	Juvenile
26	Tanah Rekah	Male	Juvenile
27	Tanah Rekah	Female	Adult
28	Tanah Rekah	Female	Adult
29	Tanah Rekah	Female	Adult
30	Tanah Rekah	Female	Adult
31	Tanah Rekah	Female	Adult
32	Tanah Rekah	Female	Juvenile
33	Tanah Rekah	Female	Adult
34	Tanah Rekah	Female	Juvenile
35	Tanah Rekah	Female	Juvenile
36	Tanah Rekah	Female	Adult
37	Tanah Rekah	Female	Juvenile
38	Tanah Rekah	Female	Juvenile

Table 2. Reference to mitochondrial DNA in several cattle species

Accession number	Species	Strains	Localisation	References
AB915322	*Bos javanicus* lowi	Borneo banteng	Malaysia	Matsubayashi et al. (2014)
JN632605	*Bos javanicus* Card4	Banteng	Cambodia	Hassanin and Ropiquet (2007)
JN632606	*Bos javanicus* CERZA	Javan banteng	Indonesia	Hassanin and Ropiquet (2007)
NC005971	*Bos indicus*	Zebu cattle	Brazil	Tripathi et al. (2011)
NC006380	*Bos grunniens*	Domestic yak	China	San et al. (2007) *
NC006853	*Bos Taurus*	Taurine cattle	Korea	Chung and Ha (2004) *
NC013996	*Bos primigenius*	Aurochs	Ireland	Edwards et al. (2010)
NC024818	*Bos gaurus*	Gaur	France	Hassanin et al. (2012)
NC025563	*Bos mutus*	Wild yak	China	Na et al. (2014)
NC036020	*Bos frontalis*	Gayal	China	Wang and Zeng (2017)

Note: (*) personal communication

The results of nucleotide sequencing were edited using Bio Edit version 7.2.5 program. The edited DNA sequence was aligned using Clustal W included in the MEGA version 6.0 program (Tamura et al. 2013) with several DNA sequences from the Bovidae group published in GenBank (http://ncbi.nlm.nih.gov). All DNA sequences were paralleled by involved several homologous DNA references in public data (NCBI) (Table 2).

The analysis included calculating nucleotide composition and genetic distance based on the D-loop segment. The calculation value of genetic distance was based on the Kimura-2-parameter (K2P) substitution model. The phylogeny tree was reconstructed using the bootstrapped Neighbor-Joining (NJ) method with 1000 repetitions (Nei and Kumar 2000).

RESULTS AND DISCUSSION

Amplification and sequencing products

There were 32 samples obtained from two locations of study, Way Krui (WK), Lampung and Tanah Rekah (TR), Bengkulu. The amplified *D-loop* sequence showed varying results. The result lengths were around 740 bp for pesisir samples, except for pesisir cattle samples of WK 01 and WK 13 that were around 886 bp. Some of the sample numbers were not included in the next analysis due to it was not had good enough quality of bands. The sample was assumed will produce less validity of nucleotide sequence. From sequencing results, there were found that the amplified *D-loop* sequence showed varying results. The results of nucleotide sequences of pesisir cattle were according to the primer design, AF 22 – AF 23 consisted of 291 bp Cyt b gene, 70 bp of tRNA Thr gene, 65 bp of tRNA Pro gene, and 740-886 bp of *D-loop* (Figure 1).

The result lengths were around 740 bp for pesisir samples, except for pesisir cattle samples of WK 01 and WK 13 that were around 886 bp. The length of *D-loop* section that was used in the alignment process with related species was around 740 bp for pesisir cattle sample TR and WK, but for sample WK 01 and WK 03 were around 886 bp.

The average of nucleotide compositions in pesisir cattle samples from both locations consisted of 25.8% T base, 25.9% C base, 33.5% A base, and 14.8% G base. The most basic components that were found in pesisir cattle were A and T base with 59.3%, while G and C base was 40.7%. The base sequence of four fragments was divided into conserve and variables. Different bases consist of two types called parsimony bases and single (singleton) bases (Table 3).

Nucleotide mutations and phylogeny analysis based on the *D-loop* sequence

D-loop sequence of pesisir cattle had various lengths. The variation was due to insertion and deletion process. Within pesisir cattle, we found base nucleotide with a length of 22 nt that had tandem repeat repetition. The repeated motives that appeared were GTACATAATATTA and ATGTAATAA. Repeated motives that appeared in genus *Bos* were always started with GTAAT. Repeat segments were found in species *B. javanicus* and Lampung WK 01 and WK 03 samples. Repeat segments in *B. javanicus* and pesisir cattle sample of WK 01 were repeated for eight times, while in pesisir cattle sample of WK 13 were repeated for seven times. There were no repeated segments in *B. indicus* and other samples of pesisir cattle such as pesisir cattle number WK 19, TR 21, and TR 29 (Figure 2).

Substitution, both transitions, and transversion, often occurred in *D-loop* sequence. The phylogeny topology based on the *D-loop* sequence showed there were two groupings of pesisir cattle against *B. indicus* Pesisir cattle that were grouped with *B. indicus* had 66% bootstrap. Pesisir cattle sample of WK 01 and WK 13 were grouped with *B. javanicus* CERZA showed 100% bootstrap for WK 13 and 83% for WK 01. Other than WK 01 and WK 13 sample, there were no specific grouping patterns in the formed phylogeny tree. Pesisir cattle sample of WK and TR codes tend to gather randomly between locations groups.

Table 3. The same number of bases and different bases in the four mtDNA segments

Fragments	Conserve bases	Different bases	
		Parsimony	Singleton
Cyt B	234	45	11
tRNA Thr	63	6	0
tRNA Pro	59	6	0
D-loop	680	140	39

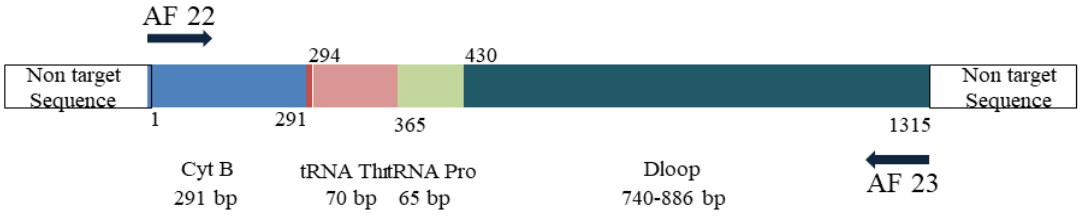

Figure 1. Products resulting from tracing based on primary design AF 22-AF 23 (bp) base pair

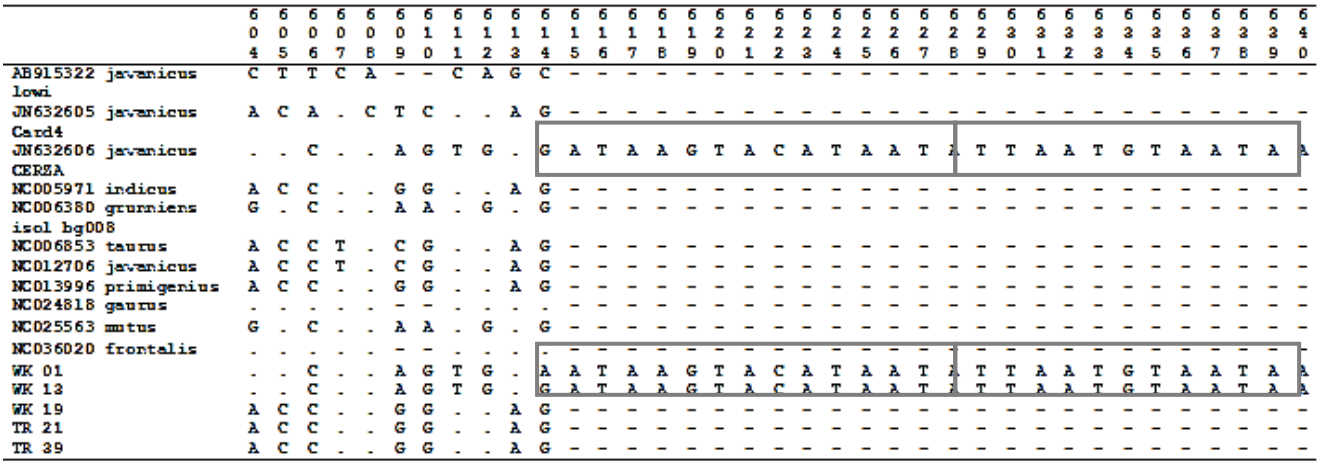

Figure 2. The section marked with a gray box is a part of a repetitive segment along 22 nt (nucleotides). Description: the first three lines indicate the position number of the nucleotide read vertically, a reference from *B. javanicus* (AB915322)

Based on nucleotide sequences, two groups of pesisir cattle can be distinguished by the appearance of insertions/deletions on 16 bases (base of 15430-15446) at the beginning of *D-loop* sequence. These were only found on WK 01 and WK 13 sample and *B. javanicus CERZA*. The insertions/deletions were also found in the base of 15485, 15516, 15615-15763, and 15793-15816. In the *D-loop* sequence, the smallest genetic distance based on the Kimura 2 Parameter model were found *B. indicus* and several TK and WK samples with a value of 0,000. The most significant genetic distance values were found between *Bos taurus* with WK 01 (0.140) and WK 13 (0.142).

Morphological observation of pesisir cattle

Morphological observations were taken at two locations, Way Krui (WK), Lampung and Tanah Rekah (TR), Bengkulu. Samples showed similar qualitative properties even though the color of samples tends to vary. The dominant color that was found in samples are brownish-white and yellowish-brown, other colors that were found are blackish-brown and white.

In general, the color pesisir cattle body was predominantly red with varieties of color ranging from yellowish to blackish. Pesisir cattle had blonde eyelashes, blackish-brown color on its backline, and whitish color on its leg. The hair on pesisir cattle tail was black. Pesisir cattle had a small body shape with small gumba and wattle. The shape of its horn is small and had small ears that pointing on the sideways.

Discussion

Amplification and sequencing products

Sequencing process was successfully carried out on samples from Way Krui, Lampung and Tanah Rekah, Bengkulu. Data visualization used 6% of polyacrylamide gel produced a double band with varied lengths. The varied length was caused by differences in nucleotide length of the *D-loop* due to repeated segments and deletion. This caused the size of the band of other pesisir cattle samples to be shorter than WK 01 and WK 13 sample.

There were several mutations in the DNA sequence of pesisir cattle, and those were deletion, insertion, and substitution (transversion and transition). The mutations were indicated by a fewer number of different bases in *Cyt b* compared to the *D-loop* sequence. Table 1 showed that nucleotides in *Cyt b* had less substitution compared to substitutions in *D-loop* sequence. This showed that the *D-loop* sequence of pesisir cattle had a high mutation rate, while the *Cyt b* segment was more stable.

Another mutation that was found in the DNA sequence of pesisir cattle was tandem repeat segments. Repeated segments caused size variations in the *D-loop*. Repeated segments of 22 nt (nucleotides) with GTACATAATATTAAT and GTAATAA motives were found in WK 01 and WK 13. Pessole et al. (1999) stated that the number of repetitions and repetitive motive segments could differ between individual and species. Repeated segments were not shared by all mammalian species. Repeated segments with different motives had been found by Nijman et al. (2003) in Madura cattle with ATTACATTAATATATGTACTT repeated twice.

The repeated segment of D-loop in Bovidae families have been documented in *Ovis aries* along 75-76 nt in the *D-loop* segment (Hiendleder et al. 2002). The function of the repeated segment in *D-loop* was not known. The existence of repetitive segments tends to reduce the size of the genome (Avise 1994). Repeated segments also formed a strong coil structure (hairpin) this structure could inhibit the action of polymerase enzyme in vitro (Gemmel et al. 1996; Farajallah 2005). The structure of the coil rod was estimated to be one of the factors that cause low success during the amplification process.

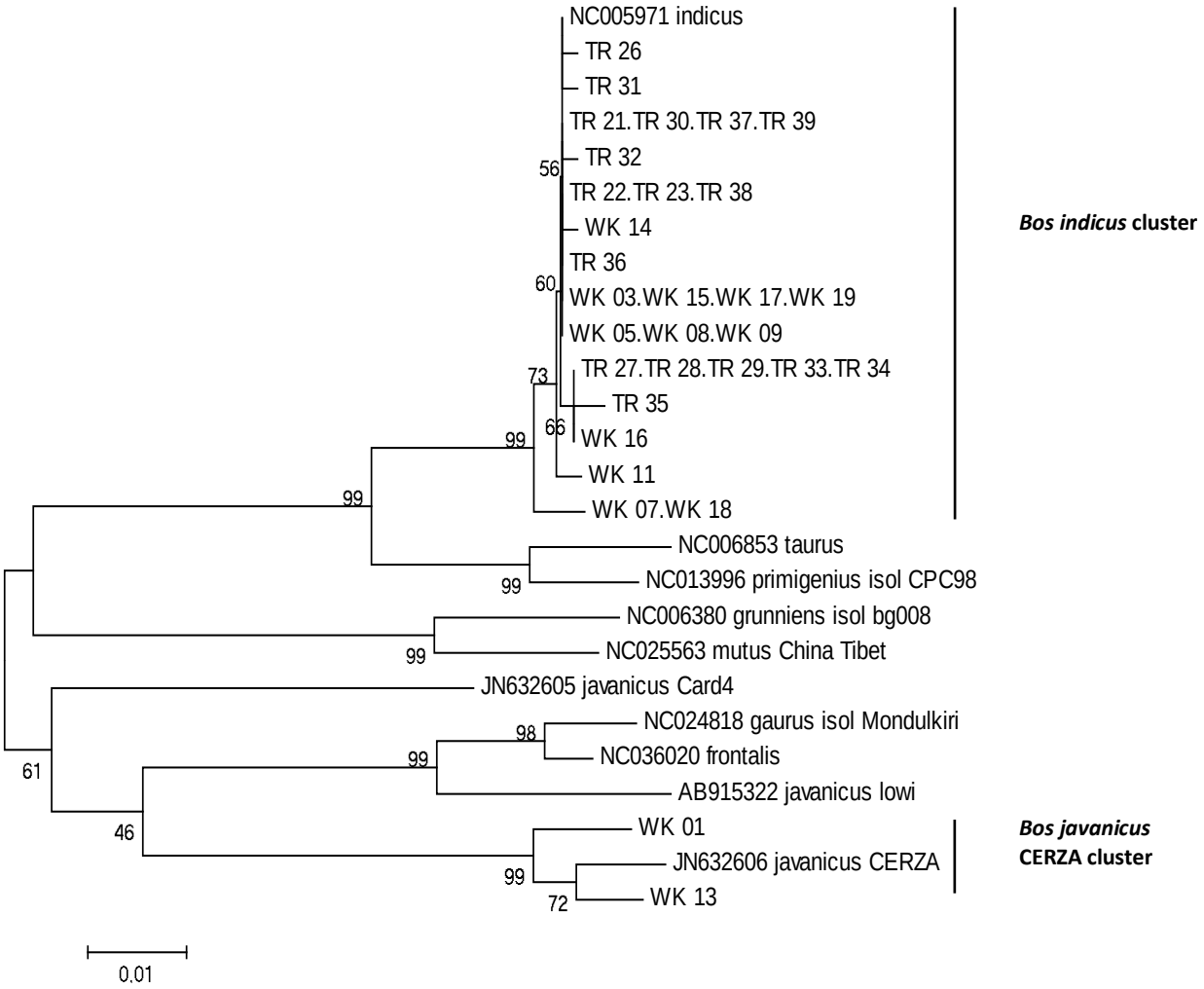

Figure 3. Results of reconstruction of phylogeny trees based on *D-loop* sections using the NJ method with 1000x bootstrap

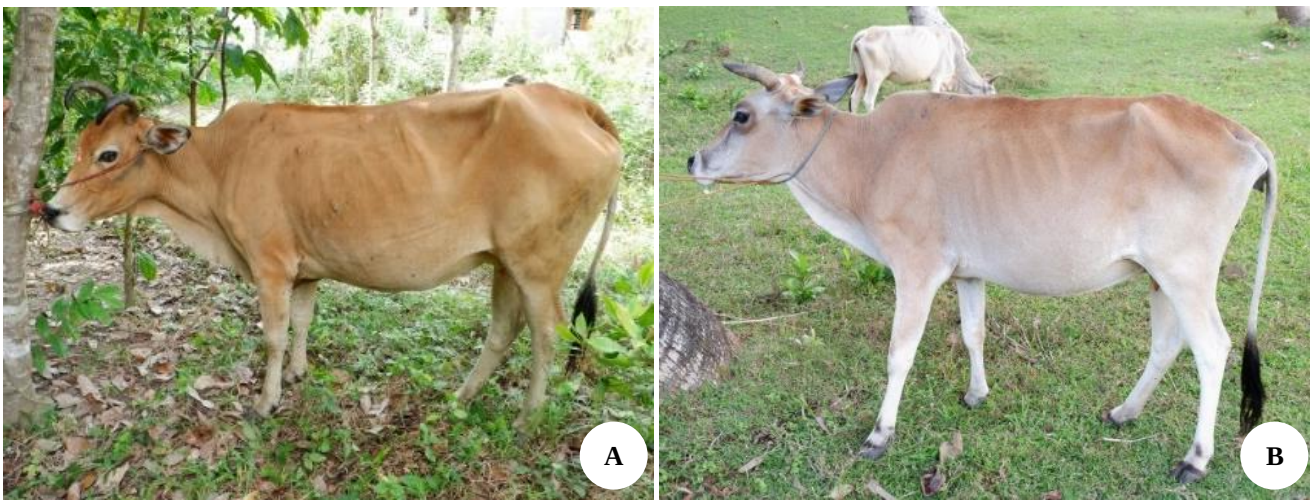

Figure 4. A. Pesisir cattle Way Krui 01. B. Pesisir cattle Way Krui 13

Nucleotide mutations and phylogeny analysis based on the D-loop *section*

The result of phylogeny tree reconstruction using high *D-loop* mutation rates showed samples of pesisir cattle were divided into two different groups in different branches. Pesisir cattle were clustered in one branch with *B. indicus* (Figure 3). This result supports the analysis study of pesisir cattle kinship using mitochondrial DNA was carried out by Mohammad et al. (2009) that showed the same pattern. *Cyt b* gene study showed that pesisir cattle from West Sumatra region were domesticated from *B. indicus* (Hartatik et al. 2015). This study showed that pesisir cattle have a close kinship with Aceh cattle. *D-loop* gene sequence study on Aceh cattle showed that Aceh cattle had a close kinship with *B. indicus.*

Pesisir cattle with sample number WK 01 and WK 03 were grouped with *B. javanicus* (Figures 3-4). *B. javanicus* sequence was from Javan banteng at Cerza Zoo, France. These data supported the results of the study that was conducted by Nijman et al. (2003), which stated that there were two types of pesisir cattle mtDNA based on the *Cyt b* segment, they were mtDNA *B. javanicus* and *B. indicus.* The appearance of two female ancestors was probably due to the small success rate of a cross between *B. javanicus* and *B. indicus*. The success rate of crossing between *B. javanicus* and *B. indicus* was around 70%. The crossing between *B. javanicus* and *B. indicus* was estimated to occur since the entry of Hindu culture brought by Indians to Indonesia. Pesisir cattle were found in the western part of Sumatra, and geographically close to Java, possibly *B. indicus* and *B. Javanicus* interbreed and produced pesisir cattle with both mixed genotypes.

Morphological analysis of pesisir cattle

Observations of pesisir cattle were taken at two locations, Way Krui, Lampung and Tanah Rekah, Bengkulu. Samples showed relatively similar qualitative properties, even though the color of samples tends to vary. The dominant color that was found in the samples was brownish-white and yellowish-brown. Other colors that were found were blackish-brown and white (Appendix 3). The physical appearance that possessed by pesisir cattle were also found in *B. indicus* and some in *B. javanicus.* Color variations of pesisir cattle were one of the features that were also seen in *B. javanicus*, with red brick color.

Diverse colors of pesisir cattle do not found in Bali cattle, Madura cattle, and Ongole fillials (*Peranakan Ongole*). However, the diverse colors are relative resembles colors on Aceh cattle in Sumatra (Abdullah et al. 2008). From the patterns and kinds of colors, the results of this study almost the same as the results of Namikawa et al. (1980) which reported that Sumatran cattle (Aceh and pesisir cattle) have varieties of black, blackish-brown, yellow-brown, and white gray dominated by yellow-brown, and the Madura cattle have three of the same color as the Sumatran cattle those are black, yellowish-brown, and white-gray with yellowish-brown as dominant color.

In accordance with the genetic and phenotypic analysis of Aceh cattle which were thought to be closely related to pesisir cattle (Hartatik et al. 2015), it is assumed that pesisir cattle are domesticated from *B. indicus* and then was crossed with *B. javanicus.*

This study showed that there was a mixture of origin of pesisir cattle based on the matrilineal line. Pesisir cattle were grouped into two types they were *B. indicus* and *B. javanicus.* The results of this study did not show the closeness between pesisir cattle and *B. taurus.*

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