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Polymorphism in the Mitochondrial Cytochrome B of Crossbred Madura-Limousine Cattle

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Abstract: Madura cattle breeders in rural Indonesia prefer to request artificial insemination services for their cows with Limousin bull's freeze-thawed semen. However, crossbred Madura-Limousin (Madrasin) bulls reported a high infertility rate. The cytochrome b (Cytb) of mitochondrial DNA (mtDNA) have been linked to certain types of male infertility.This study aimed to identify the mutation Cytb mtDNA in Madura-Limousin crossbred cattle to develop a fundamental breeding approach that would promote animal protein production and preserve the genetics of purebred Madura cattle. Blood samples were collected from the two bulls of crossbred Madura-Limousin, purebred Madura, and Limousin bulls for DNA analysis. The polymerase chain reaction was used to amplify the Cytb mtDNA, which was then sequenced using the Sanger method. The MEGA 7.0 software was used with the neighbor-joining method to construct the phylogenetic tree. Compared to the purebred Madura cattle (n= 2) and purebred Limousin bulls (n= 2), the crossbred Madura-Limousin bulls (also known as Madrasin) (n= 3) exhibited alterations in their nucleotide sequence of 13 nucleotide mutation between Madura compared to madrasin, and 14 nucleotide mutation between Limousin compared to Madrasin cattle. As observed in the resulting clades, the purebred Madura and Limousin cattle were grouped, while Madrasin crossbred cattle were separated. It could be concluded that Madrasin bulls exhibited alterations in their nucleotide and protein sequences of Cytb mtDNA, placing them in a distinct group from purebred Madura cattle and Limousin bulls.
Keywords: Madrasin cattle, deletion, smallholder farmers, transtition, transversion.

1. Introduction

Madura cattle (*Bos indicus*) are local beef cattle reared in natural isolation under the environmental influences of Madura Island, Indonesia. Madura cattle is reported to be the product of mating between Zebu (*Bos indicus*) and Wild banteng (*Bos javanicus*) which hybridization had happened more than 1500 years ago (Widyas et al., 2019). Madura cattle have a uniformity of characteristics in comparison to other Indonesian cattle. For about 15 centuries, natural selection and the strict environment have resulted in the breed's very high adaptability to the environment (Widi et al., 2014). Farmers rear Madura cattle as a form of savings, for additional income, as a manure producer, for social status, and cultural value (Widi et al., 2015). Over the past few decades, Madura cattle farmers have favored crossbreeding their diminutive cows by implementing artificial insemination strategies utilizing frozen semen derived from European beef varieties like Simmental and Limousin (Agustineet al., 2019). Farmers on Madura Island, where Madura cattle originate, prefer frozen Limousin bull semen over frozen Madura bull semen, with an increasing demand trend each year (Kutsiyahal, 2018). Limousin cattle are a famous breed in Indonesia used for frozen semen production by Artificial Insemination Centers (Putra et al., 2020).

Introducing exotic livestock is essential to intensifying livestock production (Marshall, 2020). Crossbreeding local cattle with imported cattle has become Indonesia's dominant livestock breeding strategy and is expected to benefit farmers and increase national production (Widi et al., 2015). Crossbred cattle may have higher meat production and market prices than native cattle in Indonesia (Putra et al., 2020). In Brazil, crossbred cattle are more efficient than purebred cattle because of their weight at birth and maturity, earlier maturation, and heavier body weights at weaning (Mendonça et al., 2020). Limousin crossbreeds display better weight gain than Madura cattle (Meles et al., 2022). The average adult Madura-Limousin crossbreed weighs 406.61 kg (Hartatik et al., 2009), compared to the purebred Limousin bull weight of 688.72 kg (Vlasova et al., 2020) and the Madura bull weight of 277.35 kg (Hartati and Putra, 2021). Crossbred Madura-Limousin bulls have a high infertility rate; since a single male's semen is utilized for breeding with several thousand females, it can create a impact (Kumaresan et al., 2021). In beef cattle production, fertility is regarded as a highly significant trait from an economic standpoint. However, infertility has been identified in tropical area as a prevalent issue, specifically among crossbred bulls of Taurine and Indicine breeds. (Muhammad et al., 2015), as is the case of Bos taurus x Bos indicus bulls (Prakash et al., 2021). This infertility can be traced genetically (Saleh Jaweesh et al., 2021), since crossbreeding introduces superior genetics to local cattle (Mendonça et al., 2020). New genomic techniques have been created to pinpoint marker haplotypes to identify fertility (Taylor et al., 2018).

Mitochondrial DNA (mtDNA), and more specifically, cytochrome b (Cytb) and cytochrome oxidase I (COI), are the methods for identifying species in most cases (Syakalima et al., 2016). The Cytb gene is one potential marker for species identification, taxonomic and phylogenetic studies. Since mtDNA has a higher copy number than nuclear DNA, its locus is ideal for DNA analysis (Rahmatullaili et al., 2019). No reports have analyzed the Cytb mtDNA of Madura-Limousin crosses or purebred Madura and Limousin cattle.

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Therefore, the aimed to determine the nucleotide alignment, position and type of nucleotide base mutation, protein alignment, genetic distance value, and phylogenetic tree based on the Cytb mtDNA gene in F1, F2, and F3 of Madura-Limousin (Madrasin) crossbred cattle compared to purebred Madura and Limousin cattle.

2. Materials e Methods

The study was conducted from March to September 2021. The bulls were reared in the Bangkalan regency, East Java, Indonesia. Bangkalan regency (7°02'43.80"S, 112°44'6.36"E). Bangkalan's weather is generally hot and scorching throughout the year (24–33°C), with a relative humidity of 79%–82%, rainfall of 8–116 mm, and 4.2–24 rainy days per year (MCGA, 2022). The DNA extraction was conducted at Biomolecular Laboratory, the Faculty of Veterinary Medicine, Universitas Airlangga laboratory. Further laboratory work was conducted at the Veterinary Disease Investigation Center, Denpasar, Bali, Indonesia.

3. Animals

This study used three heads of crossbred Madura-Limousin (local name: Madrasin) bulls (n=3), two heads each of purebred Madura cattle and Limousin cattle (n=2, respectively). Madrasin cattle are the offspring of Madura cows inseminated with frozen-thawed Limousin bull semen. All bulls were aged 3–5 years and were given elephant grass (*Pennisetum purpureum*) equivalent to 10% of their body weight, along with 9 kg of concentrate containing 16%-17% crude protein, and had access to drinking water.

4. Mitochondrial DNA extraction

The bovine genomic mtDNA was isolated from the bulls' whole blood cells; 10 ml blood samples were taken via venipuncture with a 10 ml project tube containing 10% EDTA. A 200 µl buffer (AL) containing Carrier RNA and 20 µl proteinase was added to 200 µl of the blood sample samples, then vortexed for 15 s. Following sample collection, they were incubated at room temperature for 10 minutes, then centrifuged for 15 seconds to eliminate water droplets from the cap. Next, 200 µl of ethanol (96%–100%) was added and vortexed for 15 seconds. Afterward, 620 µl of the resulting mixture was added to the 2 ml QIAamp mini spin column (Qiagen) and centrifuged at 6000 × g for 1 minute. The filtrate was discarded, and the QIAamp mini spin column was transferred to a new 2 ml collection tube. This process was repeated before adding 500 µl Buffer AW1, followed by centrifugation at 6000 × g for 1 minute. After the filtrate was discarded, the QIAamp mini spin column was placed into a new 2 ml collection tube. The QIAamp mini spin column was opened carefully, and 500 µl Buffer AW2 was added before being centrifuged at 20,000 × g for 3 minutes. Finally, 200 µl Buffer AE was added, incubated at room temperature for 5 minutes, and centrifuged at 6000 × g for 1 minute (Saleh Jaweesh et al., 2022). Finally, the extracted DNA samples were freeze-dried (Straube and Juen, 2013) for polymerase chain reactions (PCR) and sequencing.

5. Amplification of mitochondria DNA in Cytb region

A PCR machine (Thermocycler) was used to amplify the d-loop region of the mtDNA in the obtained DNA extract. In summary, a combination of 12.5 µl GoTaq® Green Master Mix (Promega, Madison, USA), 1 µl BIDL-F primer, 1 µl BIDL-R primer, 8.5 µl Nuclease Free Water, and 2 µl DNA template was added to a 0.1 ml PCR tube (Larrea-Sarmiento et al., 2019). Each sample was processed in four replicates. The primers employed were: 5'GCAATTGCCATAGTCCACCT'3 (Cytb_F) and 5'GGATTTGCCGGGGTATAGTT'3 (Cytb_R) (Uni Prot KB-P00157 (CYB_BOVIN) (https://www.uniprot.org/uniprot/P00157#names_and_taxonomy; February 10, 2021). To initiate amplification, 1 µL of DNA, 1 µL of forward primer, 1 µL of reverse primer, 5 µL of PCR mix (containing dNTPs, Taq polymerase, MgCl2), and 2 µL of ddH2O were mixed in a 200 µL microtube. The subsequent stages included predenaturation at 94oC for 2 minutes, followed by denaturation at 94oC for 30 seconds, annealing at 60oC for 30 seconds, extension at 72oC for 1 minute, and post-extension at 72oC for 7 minutes. These steps were repeated for up to 35 cycles. The DNA amplification product was subsequently analyzed using 2% agarose gel electrophoresis and stained with nucleic acid gel dye (Lorenz, 2012).

6. Electrophoresis

DNA was visualized via electrophoresis in a horizontal bath using 2% agarose gel. It was made by dissolving agarose in a 1X TBE buffer and heating it in a microwave for ±30 s until it was homogeneously mixed. The agarose solution was left to cool until the temperature was ± 60□C before adding 0.2 µg/ml ethidium bromide. Subsequently, the DNA was visualized under ultraviolet light. The agarose solution was then poured into an electrophoresis bath previously installed with a molding comb and was allowed to harden for 15–20 min. Electrophoresis was performed for 30 min at 90 V, then the DNA was visualized under ultraviolet light in a dark room and imaged using Gel Doc 2000 with a red filter. Suitable DNA electrophoresis results were indicated in a qualitative test by thick DNA bands with little or no smear (Feuillie et al., 2014).

7. Sequencing

We used the single bands obtained from PCR on an agarose gel as templates for sequencing reactions using forward and reverse primers during amplification (Mustofa et al., 2021). DNA sequencing was conducted using the Sanger method. The sequence of nucleotide bases was analyzed using MEGA 7.0 software, and a neighbor-joining phylogenetic tree was constructed with 1,000 bootstrap replicates (Kumar et al., 2016).

8. Results

We obtained DNA bands with 216 bp from the PCR amplification of the Cytb mtDNA from the F1, F2, and F3 Madrasin, Madura, and Limousin bulls (Figure 1). The nucleotide Cytb mtDNA sequences from the Madura and Limousin bulls had three polymorphic sites with two haplotypes at m.6T/C, m.18C/T, and m.55C/T, consist of 15 base pairs fragment of nucleotides (Table 1 and Figure 2). All crossbred Madrasin F1, F2, and F3 bulls had identical nucleotide sequences; however, there were deletions, transitions, and transversions of the Madrasin (F) nucleotide sequences compared to the Madura (M) and Limousin (L) bulls. There were 13 nucleotide mutation M>F, dan 14 nucleotide mutation L>F. Among these mutations, there were 12 same nucleotide mutations at the same position in M>F as in L>F, consisting of a deletion of A, two transitions of A to G, and of C to T, two transitions of G to A, three transitions of T to C , a transversion of A to C and of C to G. The difference is transition C to T in M>F, and transition C to T and transition T to C in Mutation L>F, but each is in a different position (Table 2 & Figure 2). These nucleotide mutations were followed by three amino acid fragment mutation, consist of the deletion of isoleucine (position number 11), histidine (position number 17) to tyrosine, and mutation of isoleucine (34) to methionine (Figure 3).

Our analysis revealed that the purebred Madura and Limousin cattle belong to a single clade, while the Madrasin crossbreed cattle are placed in a distinct clade. The purebred clade's bootstrap confidence values were 100, and the bootstrap confidence values for Madrasin crossbred cattle with purebred parents were 96–100 (Figure 4). The genetic distance between the purebred Madura and Limousin cattle was only 0.018. In contrast, the genetic distance of the crossbred Madrasin compared to the purebred Madura and Limousin was 0.078 and 0.085, respectively (Table 3).

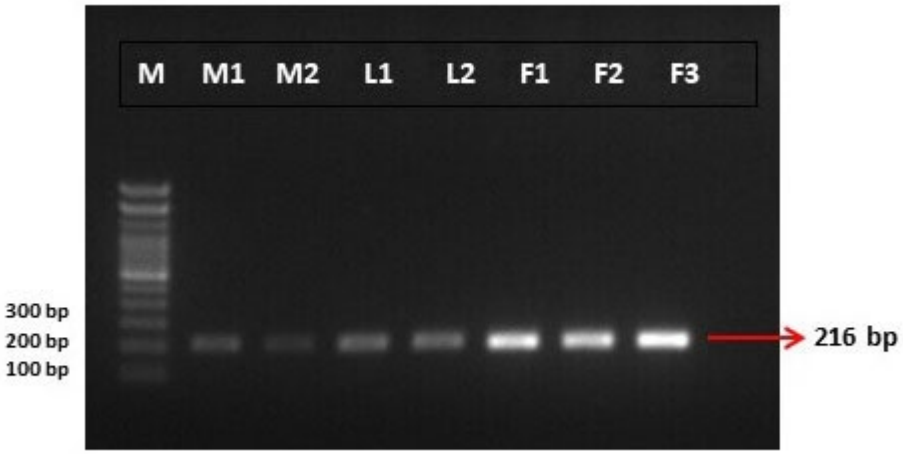

Figure 1 – Total DNA electrophoresis results of the polymerase chain reactions (PCR) product on 2% agarose gel. Note: M: Marker; M1, M2: Madura bull pure breed; L1, L2: Limousin bull pure breed; F1, F2, F3: Madrasin bull crossbreed.

No	Base position	Madura cattle	Limousin cattle	Mutation Type
1	6	T	C	Transition
2	18	C	T	Transition
3	55	C	T	Transition

Table 1 – Position and type of nucleotide base differences of Cytochrome b mtDNA of Madura Cattle and Limousin Cattle. Note: T: thymine, C: cytosine.

No	Base Position	Mutation M>F	Mutation L>F
1	6	-	Transition C>T
2	18	-	Transition T>C
3	37	Deletion A>–	Deletion A>–
4	55	Transition C>T	-
5	66	Transition G>A	Transition G>A
6	73	Transition T>C	Transition T>C
7	78	Transition G>A	Transition G>A
8	84	Transversion C>G	Transversion C>G
9	85	Transition T>C	Transition T>C
10	99	Transition T>C	Transition T>C
11	105	Transition A>G	Transition A>G
12	114	Transition A>G	Transition A>G
13	126	Transition C>T	Transition C>T
14	141	Transition C>T	Transition C>T
15	144	Transversion A>C	Transversion A>C

Table 2 – Position and type of nucleotide base mutation of Cytochrome b mtDNA of Madrasin compared to Madura Cattle (M>F), and Madrasin compared to Limousin cattle (L>F). Note: A: adenine, C: cytosine, G: guanine, T: thymine.

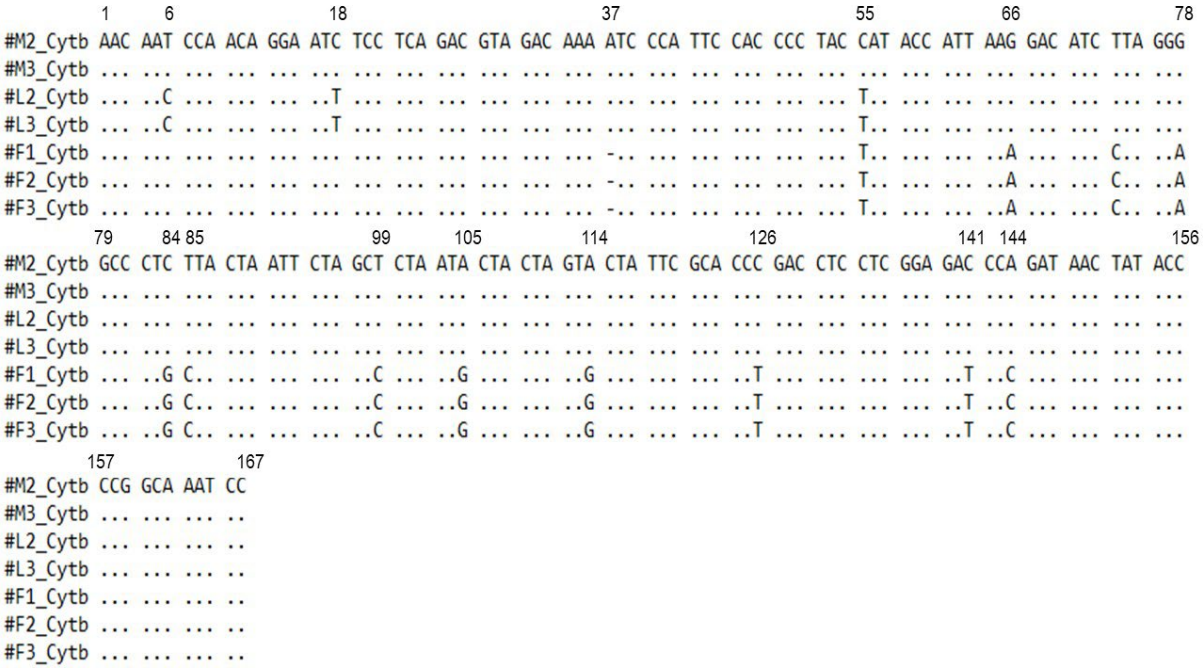

Figure 2 – Nucleotide alignment of cytochrome b mtDNA of Madura (M2 and M3), Limousin (l2 and L3), and F1, F2, and F3 of Madrasin cross breed cattle. Note: A: adenine, C: cytosine, G: guanine, T: thymine.

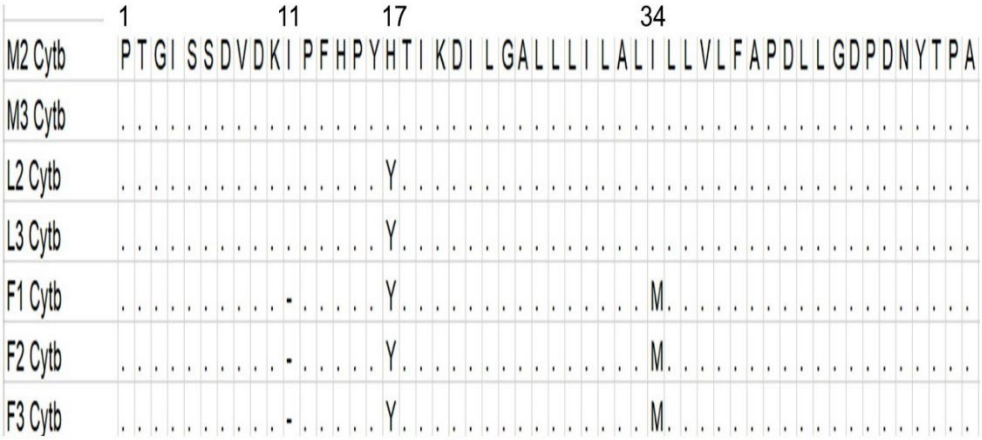

Figure 3 – Amino acid alignment of cytochrome b mtDNA of Madura (M2 and M3), Limousin (l2 and L3), and F1, F2, and F3 of Madrasin cross breed cattle. Note: Amino acid code: F = phenylalanine; H = histidine; S = serine; T = threonine; E = glutamic acid; K = lysine; G = glycine; N = asparagine; I = isoleucine; D = aspartic acid; W = tryptophan; A = alanine; R = arginine; Y = tyrosine; M = methionine.

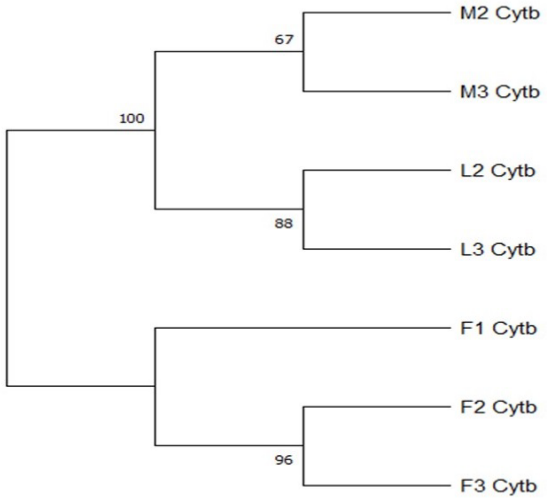

Figure 4 – Madura pure breed, Limousin pure breed, and Madrasin cross breed cattle phylogenetic tree based on cytochrome b mtDNA. Note: M2, M3: Madura bull pure breed; L1, L2: Limousin bull pure breed; F1, F2, F3: Madrasin bull cross breed.

	M2 Cytb	M3 Cytb	L2 Cytb	L3 Cytb	F1 Cytb	F2 Cytb	F3 Cytb
M2_Cytb							
M3_Cytb	0,0000000000						
L2_Cytb	0,0182128726	0,0182128726					
L3_Cytb	0,0182128726	0,0182128726	0,0000000000				
F1_Cytb	0,0779951161	0,0779951161	0,0849251061	0,0849251061			
F2_Cytb	0,0779951161	0,0779951161	0,0849251061	0,0849251061	0,0000000000		
F3_Cytb	0,0779951161	0,0779951161	0,0849251061	0,0849251061	0,0000000000	0,0000000000	

Table 3 – Values of Genetic Distance between Madrasin, Madura, and Limousin cattle based on the Cytochrome b mtDNA gene. Note: M2, M3: Madura bull pure breed, L1, L2: Limousin bull pure breed, F1, F2, F3: Madrasin bull crossbreed.

9. Discussion

Crossovers during meiotic recombination lead to genetic diversity in natural and artificial insemination (Wang et al., 2019). mtDNA has functioned as a genetic marker in intraspecies variability studies that provide qualitative and quantitative information. Among many molecular markers, mtDNA has been widely employed to predict cattle genetic diversity and phylogenetic relationships (Yan et al., 2019). The mutation rate of mtDNA is much higher than in nuclear DNA (Andalib et al., 2017).

Furthermore, Cytb mtDNA contains protein-coding genes that provide an abundance of phylogenetic intra- and interspecies information and have a higher variation ratio than other functional genes (Çiftci et al., 2013). Genetic alterations in the mitochondrial DNA (mtDNA) have been linked to certain types of male infertility and abnormal sperm function (Saleh Jaweesh et al., 2021). Hence, Cytb mtDNA is considered helpful for determining genetic diversity and phylogenetic relationships (Prihandini et al., 2020) and is also suitable for tracking natural hybridization between two subspecies and reconstructing the phylogeny of several closely related species (Merheb et al., 2019). Cytb is one of the mitochondrial protein-coding fragments involved in electron transport (Li et al., 2021). The Cytb of mtDNA plays a significant role in unraveling the population history of livestock species (Tarekegn et al., 2018), such as in Malaysian cattle breeds (Romaino et al., 2014), in Jabres, Rambon, Zebu, and Bali cattle (Sutarno Setyawan, 2016), in the genetic diversity of local Indonesian cattle (Hartatik et al., 2015), and Madura and Java cattle (Hartatik et al., 2018).

10. Nucleotide bases and amino acids mutation

Naturally, genetic diversity regulates the adaptation of a population to environmental changes, such as diseases (Freitas et al., 2021), and anticipates unpredictable population growth and climate change (Schierenbeck, 2017). Variations of mtDNA in a population are genetically inherited from their dam and bull (Tarekegn et al., 2018); thus, variation in the Cytb sequences of mtDNA can identify genetic diversity (Rahmatullaili et al., 2019). Abnormal nucleotide sequence variants can result in the loss of protein function or damage to sperm protein structure and function, which in turn reduces semen quality or increases sperm morphological abnormalities (Taylor et al., 2018). Decreased mtDNA copy numbers exacerbate mitochondrial aberrations in spermatocytes and spermatids in the testes (Jiang et al., 2017), which can lower reproductive success since mtDNA plays a vital role in gametogenesis and fertilization (Fu et al., 2021). Additionally, freeze-thawed semen increases the number of mtDNA mutations and decreases sperm quality (Mustofa et al., 2021).

The difference in nucleotides between Madura and Limousin (CAT/ TAT) was followed by the difference in amino acid number 17 of Histidine (H) in Madura and Tyrosine (Y) in Limousin cattle. These differences are natural and do not cause fertility problems. Thus, the mutation of 17 amino acids (p.H17Y) from Madura to Madrasin cattle can be ruled out as causing infertility in Madrasin cattle. The mutation of the ATA codon (in Madura cattle) to ATG (in Madrasin cattle) causes a change (p. I34M) in amino acid Isoleucine (in Madura cattle) to Methionine (in Madrasin cattle). The remaining mutations did not cause any changes in the amino acids (silent mutation). Thus, the determining factors for the impaired fertility in Madrasin cattle may be due to the nucleotide mutation followed by the deletion of number 11 amino acid (p.11I>–) and the amino acid mutation at number 34 position Isoleucine to Methionine (p. I34M). In the Madura and Limousin bulls, the 34 amino acid is the same (Isoleucine).

Infertility in the Madrasin crossbreed may be related to reproductive physiology, which involves metabolizing the mutated amino acids on reproductive hormones. The reproductive endocrine system involves several protein hormones, enzymes for the synthesis of these hormones, and their receptors (Marques et al., 2018), which include GnRH (Pérez et al., 2017), FSH (Meher et al., 2015), and LH (Cañizares-Martíne et al., 2021). Deleting number 11 amino acid p.11I>– and amino acid mutation p. I34M of cyt b mt DNA Madrasin bull may be related to the malfunction of those reproductive hormones. It needs further study to reveal this phenomenon.

11. Phylogenetic analysis

Changes in genetic diversity and population dynamics can occur in cattle populations when frozen semen from exotic bulls is introduced via artificial insemination (Sutarno Setyawan, 2016). Many developing countries compete to crossbreed their local cattle with exotic cattle to improve the production performance. In particular, livestock populations and cattle production can be increased by crossbreeding local cattle with Simental and Limousin frozen semen (Hartatik et al., 2014). The high demand for these types of semen tends to increase the farmers' livestock populations resulting from the crossing, which can shift the population dynamics of the present local livestock (Kutsiyahal., 2018).

This study generated a neighbor-joining tree to verify the phylogenetic relationship and bootstrap replications (Bunmee et al., 2018) of the Cytb genes between the Madrasin crossbreed, purebred Madura, and purebred Limousin cattle. Our analysis of the phylogenetic tree revealed that the purebred Madura and Limousin cattle share the same clade and have a genetic distance of only 0.018, while the F1, F2, and F3 Madrasin crossbreed cattle were in a different clade with genetic distances of 0.078 and 0.085 compared to the Madura and Limousin cattle, respectively. This value was 4.3–4.7 fold farther than the genetic distance value of the parents. The purebred clade's bootstrap confidence values were 100, and the Madrasin clade's bootstrap confidence values with the parents were 96–100. The percentage of bootstrap confidence values means the number of times the same values will be obtained when repeating the phylogenic reconstruction of the samples 1000 times (Hartatik et al., 2019). These findings were expected given the infertility problems of the Madrasin crossbreed cattle compared to the purebred cattle.

The expression levels of several functional proteins regulated in the spermatogenesis of crossbred bulls were related to a high incidence of infertility (Muhammad et al., 2015). Scrotal circumference, testicular vascular cone, and testicular morphology play a role in determining the testicular thermoregulatory capability and are correlated with semen quality and sperm production (Schliep et al., 2017). There is a correlation between scrotal circumference, sperm motility, and fertility, particularly in crossbred beef bulls (Kastelic et al., 2018). Crossbred sires have a higher incidence of poor semen quality and subfertility/infertility than Zebu sires (Sweett et al., 2020). Crossbred males exhibit alterations in testicular cytology indices, hormonal concentrations, sperm phenotypic characteristics, and seminal plasma composition compared to purebred males (Kumaresan et al., 2021). The crossbred bull sperm exhibited significant transcript activity associated with various functions, including the formation of ribosomal structures,

translation processes, and pathways related to ribosomes, oxidative phosphorylation, and spliceosomes (Elango et al., 2020) in the mitochondrial membrane, and estrogen signaling (Prakash et al., 2020).

This study indicates the presence of point mutations, amino acid mutations, and clade differences between crossbred Madrasin cattle with purebred Madura and Limousin parents. The findings of this study and several previous reports that indicate increased infertility in male and female crossbred cattle suggest that Madrasin crossbreed cattle should not be used for breeding. Furthermore, Madura cows are morphologically smaller than Limousin bulls (Widi et al., 2014), causing dystocia (Saraf et al., 2021). A breeding program to produce superior purebred Madura cows that use semen from elite Madura bulls needs to first result in larger Madura cows to reduce the risk of dystocia. With this strategy, the conservation of the Madura cattle can be maintained. Meanwhile, the aim of crossbreeding Madura cows with freeze-thawed semen from Limousin cattle was to produce male calves of larger size for meat production (Meles et al., 2022).

This study was limited in determining the genetic diversity and phylogenetic tree based on the Cytb mtDNA gene of Madura-Limousin (Madrasin) crossbreeds compared to purebred Madura and Limousin cattle. There has been no report on the relationship of Cytb mutations to infertility in these three cattle breeds based on their endocrine dynamics. Future studies need to be carried out on a larger cattle population to identify the correlation of Cytb mtDNA mutations with their infertility and hormonal patterns.

12. Conclusion

The nucleotide and protein sequence of Cytb mtDNA in Madrasin bulls underwent alteration, causing them to be placed in a distinct clade from the purebred Madura and Limousin cattle. This study revealed a mutation in Madrasin crossbred cattle that could be responsible for their subfertility. As a result, Madrasin crossbred cattle are deemed more appropriate for meat production rather than breeding. A breeding program of Madura cattle using artificial insemination with elite bull straw should be performed to obtain larger Madura cows, thereby reducing the risk of dystocia and simultaneously conserving Madura cattle.

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