Research Article

Identification of Myostatin (MSTN) Gene in Indigenous Pesisir Cattle

Tiara Putri Artha[1], Khasrad[2]*, Tinda Afriyani[2], Kusnadidi Subekti[2]

[1]Doctoral Student of Animal Technology and Production, Faculty of Animal Science, Universitas Andalas, Padang 25175, Indonesia; [2]Department of Animal Technology and Production, Faculty of Animal Science, Universitas Andalas, Padang 25175, Indonesia.

Abstract | Pesisir cattle are a native breed of West Sumatra that are well adapted to tropical environments, although their production performance remains lower than that of introduced breeds. This study aimed to identify genetic diversity across all exon regions of the myostatin (MSTN) gene in Pesisir cattle using the Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) method. Blood DNA samples from 96 animals were amplified using specific primers: exon 1 (F: GTTTGGCTTGGCGTTACTCA; R: GCTGATACTGTCTCCCAATCC), exon 2 (F: TCTCTGGAAAGGAAGTAGGCT; R: AGCACATCAACGGAAAGCAT), and exon 3 (F: ACTCCACAGAATCTCGATGCT; R: GGTAACTTGCGTACGGTGTG). PCR products were digested with the restriction enzyme *BfaI* to detect polymorphisms. The results showed that exons 2 and 3 were polymorphic, exhibiting two genotypes (+/+) and (+/−), whereas exon 1 was monomorphic with only the (+/+) genotype. In exon 2, genotype frequencies of (+/+) and (+/−) were 0.239 and 0.760, with allele (+) and (−) frequencies of 0.619 and 0.380, respectively. In exon 3, genotype frequencies of (+/+) and (+/−) were 0.906 and 0.093, while allele (+) and (−) frequencies were 0.953 and 0.046, respectively. Hardy–Weinberg equilibrium analysis indicated that exon 3 was in equilibrium ($X^2v < X^2t$), whereas exon 2 deviated from equilibrium ($X^2v > X^2t$). These findings indicate genetic variation in the MSTN gene of Pesisir cattle, which may contribute to differences in muscle growth performance and provide a basis for further genetic evaluation and breeding programs.

Keywords | Genetic diversity, Pesisir cattle, Myostatin (MSTN), Exon, PCR-RFLP

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***Correspondence** | Khasrad, Department of Animal Technology and Production, Faculty of Animal Science, Universitas Andalas, Padang 25175, Indonesia; **Email:** khasrad@ansci.unand.ac.id
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INTRODUCTION

The Pesisir cattle is one of the local livestock genetic resources that has been designated as an Indonesian local cattle breed through the Decree of the Minister of Agriculture of the Republic Indonesia (2011) No. 2908/Kpts/OT.140/6/2011, which plays an important role in the sustainability of national meat production. This cattle population is widely found in the coastal areas of West Sumatra and is known for its high adaptability to tropical environmental conditions, efficiency in utilizing low-quality feed, relative resistance to disease (Putri *et al.*, 2019) and fairly high carcass percentage (Yurnalis *et al.*, 2017), reaching up to 53% (Khasrad, 2006). However, its production performance, especially in terms of growth and carcass quality, is still lower than that of introduced cattle breeds such as Limousin, Simmental, and Belgian Blue (Sutarno and Setyawan, 2015). This situation indicates the need to improve the genetic quality of Pesisir cattle through a molecular-based scientific approach to identify

untapped genetic potential and support more effective and sustainable breeding programs.

One of the candidate genes that plays an important role in regulating muscle growth in livestock is the myostatin (MSTN) gene, also known as growth differentiation factor 8 (GDF8). This gene belongs to the transforming growth factor-beta (TGF-β) family, which functions as a negative regulator of skeletal muscle growth through the inhibition of myogenic cell proliferation and differentiation (McPherron *et al*., 1997). Mutations or genetic variations in MSTN can cause the loss of this inhibitory function, thereby triggering increased muscle tissue formation known as the double muscling phenotype, as observed in Belgian Blue and Piedmontese cattle (Grobet *et al*., 1997; Kambadur *et al*., 1997). Thus, characterization of the MSTN gene in local cattle, including Pesisir cattle, has the potential to provide a deeper understanding of the genetic basis of muscle growth and opportunities for its utilization in genetic selection programs.

Research on the myostatin (MSTN) gene in Indonesian indigenous cattle remains limited, particularly with respect to the populations studied and the specific gene regions analyzed. Previous studies have predominantly focused on selected segments, such as exon 1 or exon 3, despite the fundamental role of exonic regions in encoding functional protein sequences that determine the biological activity of myostatin. Single-nucleotide polymorphisms (SNPs) within these coding regions may induce alterations in protein structure and function, thereby influencing muscle growth–related phenotypes. Although, Naufal *et al*. (2024) identified polymorphisms in the MSTN gene in several Indonesian breeds, including Bali, Madura, and Peranakan Ongole cattle, comprehensive molecular information particularly across all exonic regions remains scarce for Pesisir cattle. This gap underscores the need for a systematic exploration of MSTN exonic variation in Pesisir cattle to better understand its genetic diversity and potential implications for growth performance improvement.

The Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) method is an efficient, simple, and economical molecular technique for identifying genetic variations in target genes (Nei and Kumar, 2000). This method allows the amplification of DNA fragments from the MSTN gene, which are then cut using restriction enzymes to ensure that different DNA band patterns between individuals can be observed. Although molecular information on genetic variation in Pesisir cattle is still limited, PCR-RFLP research on the MSTN gene is important for identifying genetic diversity in the Pesisir cattle population. This study can contribute to the application of marker-assisted selection (MAS) and support local livestock genetic improvement programs. This study is expected to provide important information regarding the MSTN gene genotype for development. Thus, it is important to explore the genetic variation of the MSTN gene using the PCR-RFLP method in Pesisir cattle, given the limited molecular information on their genetics. This study is expected to provide basic information on the MSTN gene genotype that can be used to develop genetic selection strategies, improve production performance, and sustainably preserve the genetic resources of Pesisir cattle.

MATERIALS AND METHODS

Ethics approval

This study was conducted in accordance with ethical standards for animal research and has received ethical clearance from the Ethics Committee of the Faculty of Pharmacy, Universitas Andalas. Ethical approval was granted under Ethical Clearance Letter Number: 4/UN16.10.D.KEPK-FF/2026.

Sample collection

This study used 96 Pesisir cattle selected by purposive sampling from smallholder farms in Koto XI Tarusan Subdistrict, Pesisir Selatan Regency, West Sumatra Province. Blood was collected from the jugular vein, placed in 3 ml EDTA tubes, and stored at -20°C until further analysis.

DNA extraction

DNA extraction from Pesisir cattle was performed at the Central Laboratory of Universitas Andalas using the Genomic DNA Mini Kit Extraction protocol. The extraction procedure consisted of several steps as follows:

Sample preparation

Approximately 200 µL of blood was added into a 1.5 mL microtube. Red blood cell (RBC) lysis buffer was added at three times the sample volume, and the tube was inverted several times. The solution was incubated at room temperature for 10 minutes and then centrifuged at 3000 x g for 5 minutes. The leukocyte pellet was subsequently resuspended in 100 µL of red blood cell lysis buffer.

Cell lysis

A total of 200 µL of GB buffer was added to the microtube, which was then mixed vigorously. The tube was incubated at 60°C for 10 minutes, with gentle inversion every 3 minutes. At the same time, the elution buffer was preheated (200 µL for each sample).

DNA binding

An equal volume (200 µL) of absolute ethanol was added to the lysate, and the tube was mixed vigorously for 10

seconds. Any visible precipitate was broken up using a pipette tip. A spin column was placed in a collection tube, and the mixture (including the precipitate) was transferred into the column. The tube was centrifuged at 15,000 x g for 5 minutes. The collection tube was disposed of and substituted with a fresh one.

Washing

The spin column was rinsed with 400 µL of buffer W1 and centrifuged at 15,000 x g for 1 minute. The effluent was dumped, and the column was reinserted into the original collection tube. Subsequently, 600 µL of Washing buffer was introduced, followed by centrifugation at 15,000 x g for 1 minute. The flow-through was removed once more, and the column was centrifuged until dry for an additional 3 minutes at 15,000 x g.

DNA elution

The desiccated spin column was relocated to a sterile microtube. Fifty microliters of warmed Elution buffer was applied directly to the center of the membrane. The column was incubated at room temperature for 3 minutes and then centrifuged at 15,000 × g for 30 seconds to elute the DNA. Then the quality and concentration of the extracted DNA were evaluated using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA).

Amplification and genotyping MSTN gene

The MSTN gene fragment was amplified by using the Polymerase Chain Reaction (PCR) technique. This investigation utilized unique primers designed with the Primer3 web software. The primer sequences used for each exon are shown in Table 1.

A total of 15 µL of PCR mixture consisting of 1 µL of DNA template, 7.5 µL of master mix, 0.75 µL of forward primer, 0.75 µL of reverse primer, and 5 µL of nuclease-free water. The amplification steps include initial denaturation (95°C/3 minutes), followed by 40 cycles consisting of denaturation (95°C/30 seconds), annealing (exon 1: 62°C; exon 2 and exon 3: 59°C/30 seconds), and extension (75°C/30 seconds), as well as a final extension (75°C/5 minutes) on a thermocycler (Cleaver Scientific Ltd, GTC96S). The PCR products were visualized by electrophoresis with 2% agarose (Vivantis Inc., USA) and 4 µL of Redsafe staining (iNtRON Biotechnology) at 100 V for 55 minutes. They were then visualized using a UV Transilluminator (Invitrogen by Thermo Fisher Scientific iBright 1500).

The MSTN gene was analyzed using the PCR-RFLP method. This analysis used the *BfaI* restriction enzyme (5'-C|TAG-3') to identify the genotype. This enzyme was chosen because it is known to have a recognition site found in the tested fragment, which was confirmed through the Nebcutter V2.0 website. The amplified products of exon 1 (790 bp), exon 2 (942 bp), and exon 3 (821 bp) of the MSTN gene were digested at 37°C for 2 hours. A total of 7 µL of mixture consisting of 5 µL of PCR product, 0.9 µL of nuclease-free water, 0.4 µL of BfaI enzyme, and 0.7 µL of 10x Tango buffer was used. RFLP visualization was performed using a UV transilluminator after electrophoresis with 2% agarose concentration and 4 µL of Redsafe staining, 3 µL of RFLP product, 3 µL of 50 bp DNA ladder, and 1 µL of loading dye, which was then run at 100 V for 55 minutes.

Genotypes were determined based on restriction fragment patterns obtained after enzymatic digestion of PCR products and visualized by agarose gel electrophoresis. Individuals showing only the wild-type allele were classified as homozygous (+/+), those carrying both wild-type and variant alleles as heterozygous (+/−), and those showing only the variant allele as homozygous mutant (−/−). This notation follows the standard convention for bi-allelic loci and was used for subsequent allele frequency and Hardy–Weinberg equilibrium analyses.

Data analysis

Genotype frequency indicates the proportion of a particular genotype in a given population, determined by comparing the presence of that genotype in the entire population. Meanwhile, allele frequency indicates the proportion of a particular allele compared to the total number of alleles present in a population (Noor, 2010).

Table 1: Primer sequences for exon 1, exon 2, and exon 3.

Exon	Gene	Primers	TM°	Product size (bp)
1	MSTN-F	5' - GTTTGGCTTGGCGTTACTCA - 3'	59.05	790
	MSTN-R	5' - GCTGATACTGTCTCCCAATCC - 3'	57.88	
2	MSTN-F	5' - TCTCTGGAAAGGAAGTAGGCT - 3'	57.81	942
	MSTN-R	5' - AGCACATCAACGGAAAGCAT - 3'	58.46	
3	MSTN-F	5' - ACTCCACAGAATCTCGATGCT - 3'	58.89	821
	MSTN-R	5' - GGTAACTTGCGTACGGTGTG - 3'	59.21	

Note: TM = Temperature melting.

Genotype and allele frequencies were calculated using the following formula (Nei and Kumar, 2000):

$$Xi = \frac{\sum ni}{N}$$

Explanation: xi= Observed genotype; ni = Number of individuals with genotype; N= Total sample observed.

$$Xi = \frac{2nii + \sum_{j \neq i} nij}{N}$$

Explanation: Xi = frequency of allele i, nii = number of samples with genotype ii (homozygous), nij= number of samples with genotype ij (heterozygous), N = number of samples.

Heterozygosity values were calculated using the following formula (Yeh, 1999):

$$Ho = \sum_{k}^{s} Wk \sum_{i \neq j}^{q} Xkij \; ; He = 1 - \sum_{k}^{s} Wk \sum_{i}^{q} x^2_{ki}$$

Explanation; Ho= observed heterozygosity, He= expected heterozygosity, Wk= effective population size, Xkij= frequency of genotype ij, population k.

To determine whether the allele frequency and genotype frequency are in equilibrium, $p^2 + 2pq + q^2$, the chi-square test is performed as follows:

$$X^2 = \sum \frac{(Oi - Ei)^2}{Ei}$$

Explanation: X^2 = chi-square, Oi = number of observations of genotype/allele i, Ei = expected number of genotype/allele i.

Balance is determined by comparing the X^2 value with the X^2 table. If the result of $X^2v \leq X^2t$ 0.05, then there is no significant difference between the observed and expected results, or the results satisfy the Hardy-Weinberg equilibrium. Meanwhile, if $X^2v \geq X^2t$ 0.05, then there is a significant difference between the observation and the theory, or the results do not satisfy the Hardy-Weinberg equilibrium.

RESULTS AND DISCUSSION

DNA EXTRACTION

A total of 96 DNA samples from Pesisir cattle were successfully extracted from blood utilizing the Geneaid DNA Extraction Kit technique. The DNA quality assessment indicated that the extracted DNA exhibited high purity, with an average value between 1.7 and 1.9 ng/µL.

The integrity of genomic DNA is a crucial determinant affecting the efficacy of PCR amplification. DNA is deemed pure when its purity ratio falls between 1.8 and 2.0 ng/µL (Muladno, 2010). This investigation revealed that certain DNA samples exhibited purity levels < 1.8 ng/µL, suggesting potential contamination by protein or phenol, corroborating the results of Ludyasari (2014). Conversely, DNA purity values over 2.0 ng/µL may suggest RNA contamination (Farrel, 1993).

MSTN GENE AMPLIFICATION

The forward and reverse primers used in this study were designed based on the myostatin gene sequence available in GenBank with accession number NC_091761.1, resulting in DNA sequences with lengths of 790 bp, 942 bp, and 821 bp for exons 1, 2, and 3, respectively. The results of the amplification of the MSTN gene sequences of exons 1, 2, and 3 in Pesisir cattle are shown in Figure 1.

Notwithstanding the discrepancies in DNA purity, amplification of the MSTN gene across all three designated exons was effectively accomplished, as demonstrated by the appearance of distinct DNA bands corresponding to the anticipated fragment sizes. Settani *et al.* (2006) assert that the presence of a singular band at the designated size verifies effective amplification, signifying that the DNA quality was adequate for subsequent PCR-RFLP analysis.

MSTN GENE POLYMORPHISM

Identification of whole MSTN gene diversity in Pesisir cattle was performed using the PCR-RFLP method with the *BfaI* restriction enzyme, which recognizes the C|TAG cleavage site. The results of cutting the MSTN gene DNA sequence in each exon are shown in Figure 2. Cutting with the *BfaI* enzyme on exon 1 produced three cutting bands with lengths of 242 bp, 264 bp, and 284 bp and produced a homozygous genotype (+/+). Exon 2 produced three cleavage bands with lengths of 339 bp, 603 bp, and 942 bp and two genotypes, namely homozygous (+/+) and heterozygous (+/-), and exon 3 produced three cleavage bands with lengths of 135 bp, 288 bp, 398 bp, 821 bp and two genotypes, namely homozygous (+/+) and heterozygous (+/-).

The genotype frequencies and allele frequencies of each exon of the MSTN gene in Pesisir cattle are shown in Table 2, and the heterozygosity values are shown in Table 3. Table 2 shows that only the (+/+) genotype is present in exon 1, while exon 2 and exon 3 have two genotypes, namely (+/+) and (+/-). The genotyping results using the *BfaI* enzyme show polymorphic results in exon 2 and exon 3 and monomorphic results in exon 1.

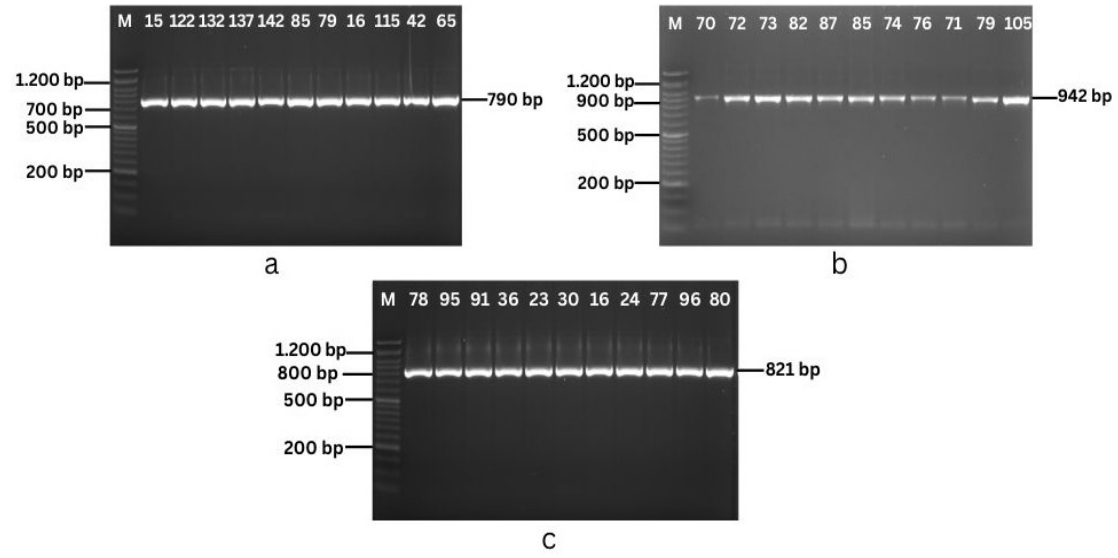

Figure 1: (a) Visualization of the amplification of the MSTN exon 1 gene with a length of 790 bp. M: 50 bp marker; 15-65: sample code, (b) Visualization of the amplification of the 942 bp MSTN exon 2 gene. M: 50 bp marker; 70-105: sample code, (c) Visualization of the amplification of the 821 bp MSTN exon 3 gene. M: 50 bp marker; 70-105: sample code.

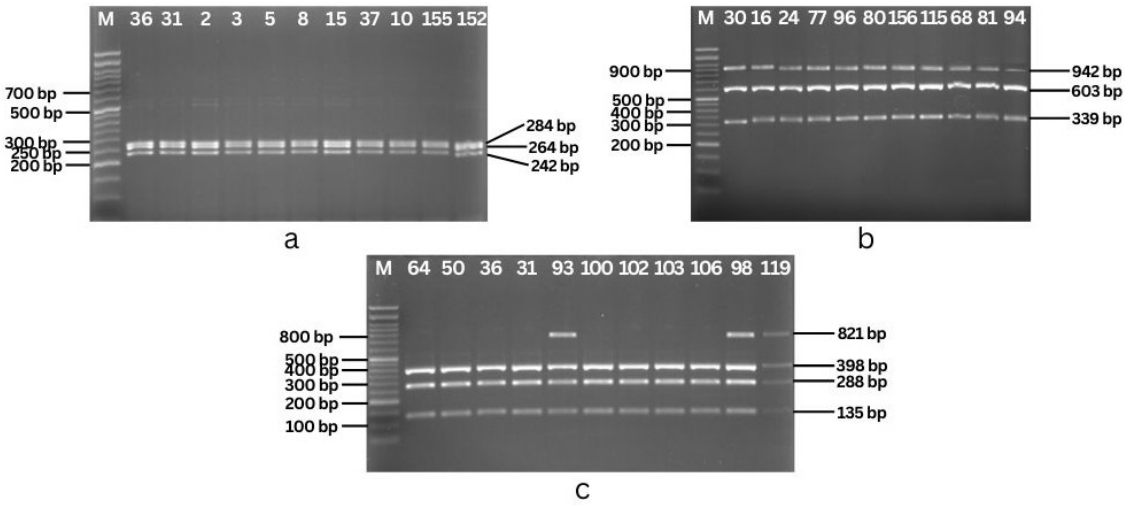

Figure 2: (a) Visualization of MSTN exon 1 restriction by the *Bfal* enzyme (CTAG). M: 50 bp marker; 36-152: sample code, (b) Visualization of MSTN exon 2 restriction by the Bfal enzyme (CITAG). M: 50 bp marker; 30-94: sample code, (c) Visualization of MSTN exon 3 restriction by Bfal enzyme (CITAG). M: 50 bp marker; 64-119: sample code.

Table 2: Frequency of genotypes and alleles of the myostatin gene in Pesisir cattle.

Exon	N	Genotype	Number of individuals	Genotype frequencies	Number of Alleles		Allele frequencies	
					+	-	+	-
1	96	(+/+)	96	1.00	192	0	1.00	0.00
		(+/-)	0	0.00	0	0		
		(-/-)	0	0.00	0	0		
2	96	(+/+)	23	0.239	46	0	0.619	0.380
		(+/-)	73	0.760	73	73		
		(-/-)	0	0.00	0	0		
3	96	(+/+)	87	0.906	174	0	0.953	0.046
		(+/-)	9	0.093	9	9		
		(-/-)	0	0.00	0	0		

Note: N = sample size.

Table 3: MSTN|Bfa1 gene heterozygosity values in Pesisir cattle.

Exon	Heterozygosity	
	Ho	He
1	0	0
2	0.760	0.473
3	0.093	0.09

Note: Ho = observed heterozygosity; He = expected heterozygosity.

The analysis of MSTN gene polymorphism demonstrated a monomorphic pattern in exon 1 and polymorphic patterns in exons 2 and 3 of Pesisir cattle. The monomorphic pattern in exon 1 likely reflects strong functional constraints, as this region contains conserved coding sequences essential for normal MSTN activity. Mutations

in conserved regions are typically removed by purifying selection, limiting detectable variation. In contrast, the polymorphisms observed in exons 2 and 3 indicate a greater tolerance for nucleotide changes or different selective pressures. Such variation may influence muscle development and contribute to genetic diversity within the population. These results align with earlier research indicating monomorphism in exon 1, including the F94L variant in Sumba Ongole cattle (Hartati *et al*., 2022) and the g.-371T>A promoter variant in Pesisir cattle (Sutikno *et al*., 2020). Conversely, Prihandini *et al*. (2021) reported divergent findings, having discovered polymorphic SNPs in exon 1 of the MSTN gene in Madura and Bali cattle. Nei and Kumar (2000) classify an allele as polymorphic when its frequency is ≤0.99, while monomorphism is defined when the allele frequency nears fixation (≤0.01). Discrepancies among research may be ascribed to variations in mutation locations, population size, breeding history, and domestication processes. According to Yuniarsih *et al*. (2011), variations in gene and allele frequencies are affected by multiple evolutionary mechanisms, such as selection, mutation, gene flow, inbreeding, outcrossing, and genetic drift.

Hardy-weinberg equilibrium

A chi-square test was performed to determine whether the data obtained in this study were in Hardy-Weinberg equilibrium. If the calculated X^2 value is greater than the X^2 table, then Ho is rejected, and if X^2 value is less than the X^2 table, then Ho is accepted. The results of the chi-square test to determine Hardy-Weinberg equilibrium are presented in Table 4.

Table 4: Hardy-Weinberg equilibrium chi-square test.

Exon	HW equilibrium	Genotype frequencies			Total	X^2v	X^2t
		(+/+)	(+/-)	(-/-)			
1	O	96	0	0	96	0	0
	E	96	0	0	96		
	(O-E)2/E	0	0	0	0		
2	O	23	73	0	96	36.21	5.991
	E	36.77	45.12	13.82	95.71		
	(O-E)2/E	5.16	17.22	13.82	36.21		
3	O	87	9	0	96	0.26	5.991
	E	87.17	8.27	0192	95.63		
	(O-E)2/E	0.000324	0065362	0.192	0.26		

Note: X^2t (0.05) = 5.991; Exon 2 = $X^2v > X^2t$ (significantly different); Exon 3 = $X^2v < X^2t$ (not significantly different).

Differences in Hardy–Weinberg equilibrium patterns were observed among the MSTN exons in Pesisir cattle, with exon 2 showing significant deviation from equilibrium, while exon 3 remained in equilibrium. This contrast suggests that exon 2 may be influenced by locus-specific genetic factors. The disequilibrium in exon 2 could be associated with selective pressures, either natural or artificial, as well as local genetic effects such as linkage disequilibrium with nearby functional variants, the presence of null alleles, or differences in mutation rates that affect genotype distribution in this region. In contrast, the equilibrium observed in exon 3 indicates a stable allelic distribution without detectable distortion. These findings highlight that different exonic regions within the MSTN gene may undergo distinct evolutionary dynamics, even within the same population and sample set.

The observed diversity of the MSTN|*BfaI* gene in Pesisir cattle corroborates prior findings that highlight the functional significance of MSTN gene variation in muscle development. Research conducted by Grobet *et al*. (1997) and Kamdabur *et al*. (1997) has shown that deletions in the MSTN gene are accountable for the double-muscling phenotype in Belgian Blue and Piedmontese cattle, underscoring the significant correlation between MSTN gene variation, muscle hypertrophy, and meat yield. The Hardy–Weinberg equilibrium study elucidates the genetic structure of the population. The Hardy–Weinberg equilibrium posits a huge, randomly mated population devoid of evolutionary influences, wherein allele and genotype frequencies stay invariant across generations, represented by the equation $p^2 + 2pq + q^2 = 1$ (Vasconcellos *et al*., 2003).

Analyzing genetic diversity is crucial for assessing the possible use of genetic markers in breeding operations. The genetic variation of the MSTN gene identified in this work may provide initial evidence for marker validation. Additional validation with more accurate methodologies, such as Sanger sequencing, is necessary to delineate the specific nucleotide alterations. Furthermore, association studies linking MSTN genotypes to economically significant variables, like body weight and growth performance, are essential to ascertain the functional relevance of the identified polymorphisms. The genetic diversity of the MSTN gene may facilitate marker-assisted selection and focused breeding strategies to enhance productivity while preserving the genetic resources of Pesisir cattle in West Sumatra.

CONCLUSION

The (+/+) and (+/-) genotypes in exon 2 and exon 3 of the MSTN|*BfaI* gene produce polymorphism, while exon 1 is monomorphic with the (+/+) genotype in Pesisir cattle. The validity of whether or not a mutation has occurred in the results of this study can be determined through sequencing methods. This study provides information for further analysis, such as the relationship between the

MSTN gene and productivity traits in Pesisir cattle in West Sumatra.

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NOVELTY STATEMENT

This study provides the first comprehensive assessment of genetic polymorphism across all exonic regions of the myostatin (MSTN) gene in indigenous Pesisir cattle using the PCR-RFLP approach, revealing exon-specific variation patterns and population genetic equilibrium status that have not been previously reported.

AUTHOR'S CONTRIBUTION

All authors contributed to the conception, analysis, and interpretation of the data, as well as manuscript preparation and approval of the final version.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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