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Identification of *FREM2* SNPs in Bali cattle for improving meat quality

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Abstract. *Pertiwi EA, Maskur, Rozi T, Muhsinin M, Ulum MF, Muharram F, Jakaria. 2026. Identification of* FREM2 *SNPs in Bali cattle for improving meat quality. Biodiversitas 27 (1): d270127. https://doi.org/10.13057/biodiv/d270127*. Bali cattle (*Bos javanicus*) are indigenous to Indonesia and valued for beef quality traits influenced by genetic variation. The *FREM2* gene is recognized as one of the genetic factors that regulate marbling scores in beef cattle. This gene has been extensively studied in various livestock breeds, but has rarely been explored in local livestock breeds in Indonesia. This study aims to identify SNPs in Bali cattle as a first step in determining genetic markers to help improve meat quality, especially in Bali cattle. Blood samples were collected from 20 unrelated Bali cattle. Genomic DNA was extracted via the phenol-chloroform method. A 662 bp fragment encompassing exon 6 and intron 7 of the *FREM2* gene was amplified by Polymerase Chain Reaction (PCR) and sequenced bidirectionally using Sanger technology. Sequence alignment and SNP detection were performed in MEGA X, with chromatogram inspection in FinchTV to confirm base calls. Population genetic parameters, including allele frequencies, observed and expected heterozygosity, and Polymorphic Information Content (PIC), were calculated using PopGen 3.2. Two novel SNPs were identified: g.89573C>T in exon 6, resulting in a non-synonymous Pro→Leu substitution, and g.89633G>A in intron 7. The g.89573C>T SNP deviated significantly from Hardy-Weinberg equilibrium ($\chi^2 = 4.62$, $p = 0.032$), with observed heterozygosity (Ho = 0.50) exceeding expected heterozygosity (He = 0.455) and a PIC of 0.343. The intronic g.89633G>A variant conformed to equilibrium ($p = 0.345$) with a PIC of 0.269. These *FREM2* markers enable marker-assisted selection to improve meat quality in Bali cattle while supporting breed conservation through genomic selection.

Keywords: Bali cattle, breeding, *FREM2* gene, genetic markers, meat quality, SNP

Abbreviations: *FREM2*: FRAS1-Related Extracellular Matrix 2, SNP: Single Nucleotide Polymorphism, PCR: Polymerase Chain Reaction

INTRODUCTION

Rising population numbers and increasing public awareness about the benefits of consuming animal protein are driving the growth in national beef consumption (Indra 2021). Indonesia is a country with a fairly high genetic diversity of livestock, as recorded in the FAO's Domestic Animal Diversity - Information System (DAD-IS) database (2020), which lists approximately 206 large ruminant, small ruminant, poultry, and pig species. Bali cattle (*Bos javanicus* d'Alton, 1823) is one of the original and pure Indonesian beef cattle that has received a lot of attention from various parties because it has superior characteristics compared to other native cattle. Bali cattle exhibit high adaptability, enabling them to spread throughout nearly all of Indonesia (Aritonang et al. 2017). Bali cattle, an indigenous breed of Indonesia, possess significant potential to produce high-quality beef compared to other local and imported breeds (Tahuk et al. 2018). Bali cattle have superior meat quality with low fat content (Agustina et al. 2021).

Beef quality is determined by factors such as palatability (tenderness, moisture, flavour) and visual characteristics (meat colour, fat colour, marbling). Among these attributes, marbling score the distribution of intramuscular fat within muscle tissue—is considered one of the most critical determinants of beef quality (Tian et al. 2024). Identifying genetic markers associated with these traits is crucial for the development of effective breeding strategies and the implementation of marker-assisted selection programs.

The identification of the cattle genome represents a breakthrough in the development of genetic tools that have significantly contributed to advancements in cattle breeding over the past several decades (Puga-Torres et al. 2021). Livestock selection programmes using Marker-Assisted Selection (MAS) can be effective if the genetic markers used as the basis for selection show sufficient diversity (Agung et al. 2017). Recently, Genome-Wide Association Studies (GWAS) have been utilized in domestic animal genetics and breeding, leading to the identification of numerous genetic markers linked to economically significant traits (Du et al. 2021). This Genomic prediction and Selection (GS) approach is now routinely applied in both animal and plant breeding programs to enhance genetic improvement (Sandhu et al. 2022).

Genes are the genetic material that plays a role in the inheritance of traits. Genes that affect meat quality have been widely reported by scientists, such as Calpastatin (CAST), a gene that influences meat quality in sheep (Greguła-Kania et al. 2019). The FRAS1-Related Extracellular Matrix protein 2 (*FREM2*) gene has emerged as a candidate gene of significant interest in livestock genetics research. This gene encodes a large extracellular matrix protein that forms a ternary complex with FREM1 and FRAS1 proteins, playing crucial roles in maintaining basement membrane stability, promoting cell-cell adhesion, and regulating tissue morphogenesis during development (Jordan et al. 2018). *FREM2* gene is a gene that has been widely reported in various livestock species. Previous studies have identified several functional SNPs in the *FREM2* gene in various livestock breeds. For example, a study on Hanwoo cattle by Bedhane et al. (2019) identified SNPs associated with marbling scores. Studies on other breeds found different associations. Casertana pigs showed links with neck pouches (Schiavo et al. 2019). Iberian pig crosses were associated with body weight and morphological size at birth and weaning (Óvilo et al. 2022). In Hu sheep, *FREM2* SNPs were linked to the number of teats (Zhao et al. 2022). For the first time, the *FREM2* gene was explored in Bali cattle. Results showed that one SNP was significantly associated with the thickness of the longissimus dorsi muscle (Pertiwi et al. 2024). However, *FREM2* SNPs have not been systematically characterized in Bali cattle, despite growing evidence linking this gene to meat quality traits in other breeds. The limited genomic information available for indigenous Bali cattle restricts the development of breed-specific molecular markers for MAS programs. This represents a significant knowledge gap that needs to be addressed. Discovering additional SNPs in Bali cattle is necessary to validate the role of the *FREM2* gene in meat quality.

MATERIALS AND METHODS

Animal samples and ethics approval

The study material consisted of twenty male Bali cattle, sampled from two sources: Ten from the Banjarmasin slaughterhouse and ten from the Bali Breeding Center. The study protocol received approval from the Ethics Commission of the Department of Food Security, Agriculture and Fisheries of Banjarmasin City, South Kalimantan, Indonesia, with number 520/624DKP3/XII/2021, and the ethics commission of Universitas Udayana, Denpasar, Indonesia, with number B/184/un14.2.9/pt.01.04/2021.

Procedures

Extracted DNA and FREM2 *gene amplification*

Genomic DNA was extracted using the Geneaid DNA Kit (Geneaid Biotech Ltd., Taiwan) following the manufacturer's protocol. The *FREM2* gene fragment (836 bp) encompassing exon 6 and intron 7 was amplified using the primer set: F: 5'-CAACGTCTGTGCTCTCCTAT-3' and R: 5'-AAAGAATCCACCTGCAATGC-3'. Determination of SNPs and genotypes. PCR reactions (25 µL total) contained 12.5 µL Bioline MyTaq HS Red Mix, 2 µL genomic DNA template, 0.2 µL each primer (25 pmol/µL), and 10.1 µL nuclease-free water. Thermal cycling consisted of initial denaturation at 95°C for 1 min, followed by 35 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 10 s, with a final extension at 72°C for 5 min using a Thermocycler AB System.

FREM2 PCR products were sequenced by 1st Base Selangor Malaysia using an ABI PRISM® 3730X1 Genetic Analyzer (Applied Biosystems). Reference sequence of the *FREM2* gene (Ensembl accession number ENSBIXG00005003071) was used for sequence alignment. SNP identification was performed using MEGA X (Molecular Evolutionary Genetics Analysis v10) with ClustalW alignment algorithm, followed by genotype frequency analysis using PopGen 3.2. Allele frequency and genotype frequency were calculated using the Nei and Kumar (2000) as formula follows:

$$X_i = \frac{2n_{ii} + \sum_{i \neq j} nij}{2N} \quad X_{ii} = \frac{n_{ii}}{N}$$

Where :
X_i : Allele frequency
X_{ii} : Genotype frequency
n_{ii} : Number of individuals with genotype ***ii***
nij : Number of individuals with genotype ***ij***
N : Total sample size (Nei and Kumar 2000)

Furthermore, genetic diversity is calculated according to Nei and Kumar (2000) formula, which utilizes Observed heterozygosity (Ho) and Expected heterozygosity (He) values as follows:

$$H_O = \sum_{i \neq j} \frac{N_{1ij}}{N} \quad H_e = 1 - \sum_{i=j}^{q} X_i^2$$

Where :
Ho : Observed heterozygosity value
N_{1ij} : Number of heterozygous individuals
N : Total number of individuals observed
He : Expected heterozygosity value
X_i : Frequency of a given allele
q : Total number of alleles

Hardy-Weinberg equilibrium is analyzed by Chi- Square (χ^2) according to Nei and Kumar (2000) as follow:

$$x^2 = \sum \frac{(O - E)^2}{E}$$

Where :
x^2 : Chi-Square statistic
O : Observed value
E : Expected value

The PIC value is standardized for evaluating genetic markers based on DNA bands of PCR amplification results, therefore PIC values are divided into three classes: PIC>0.5: highly informative, then 0.25>PIC>0.5: moderate, and PIC<0.25: low (Botstein et al. 1980):

$$PIC = 1 - (p^2 + q^2) - 2p^2q^2$$

RESULTS AND DISCUSSION

PCR amplification yielded specific products of 836 base pairs in all 20 samples, as confirmed by agarose gel electrophoresis in Figure 1. No evidence of non-specific amplification or primer dimer formation was observed.

SNP identification

SNP determination in Bali cattle identified two novel SNPs (Figure 2), with allele and genotype frequencies presented in Table 2.

SNP g.89573C>T is located in exon 6 at position 89,573 bp and causes an amino acid change from CCA (proline) to CTA (leucine). This substitution has the potential to alter the function of the *FREM2* protein because proline has a unique cyclic structure that provides rigidity. Replacing proline with leucine (a hydrophobic amino acid) can alter protein stability and conformation, thus affecting its ability to interact with other proteins. Whereas, SNP g.89633G>A is located in intron 7 at position 89,633 bp with an amino acid change from CTG (leucine) to TTG (leucine). While it does not alter the coding sequence, it may affect gene expression by modifying regulatory elements or splicing.

Previous studies have reported associations between *FREM2* gene variants and meat quality traits in various cattle breeds. Pertiwi et al. (2024) identified a synonymous SNP, g.89327G>A, in exon 6 of the *FREM2* gene in Bali cattle that was significantly associated with longissimus dorsi thickness. These results highlight the potential significance of this gene region for meat quality traits. The present study builds on these findings by identifying a non-synonymous variant in the same exon, which is likely to have more substantial functional effects.

In other studies, the results of SNP discovery in the *FREM2* gene in Hanwoo cattle reveal a significant association with several meat quality parameters, including marbling score, meat color, and meat texture. A Genome-Wide Association Study (GWAS) using Whole-Genome Sequence (WGS) data from 2,110 Hanwoo cattle individuals identified 107 significant SNPs spread across 14 chromosomes, with several QTLs affecting marbling on chromosomes BTA2, 12, 16, and 24, as well as QTLs affecting meat texture on BTA12 and 29. Research by Bedhane et al. (2019) and Grigoletto et al. (2020) suggests that the *FREM2* gene is a key candidate gene influencing meat quality in Hanwoo cattle. Meanwhile, research on Iberian crossbred pigs conducted by Óvilo et al. (2022) reported that Candidate genes harboring SNPs that influence weight and morphological traits during early developmental stages (birth and weaning) include ACACA, EPHX1, and *FREM2*. These genes have been associated with critical growth parameters, underscoring their importance in early development.

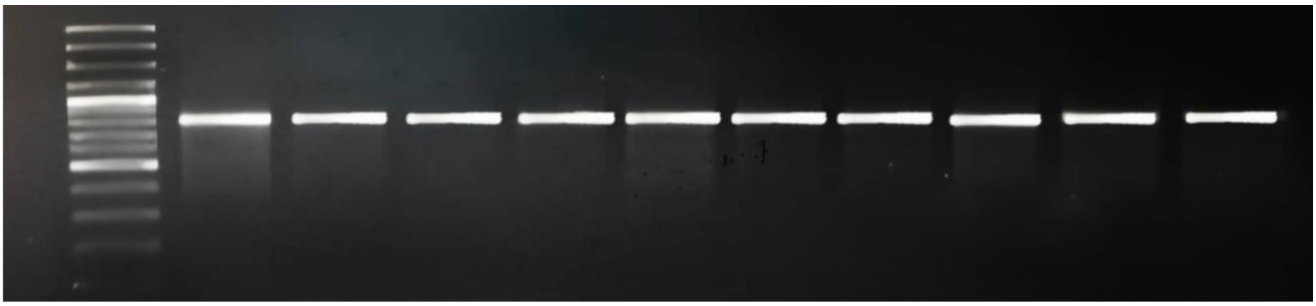

Figure 1. Agarose gel electrophoresis of PCR-amplified *FREM2* gene fragment (836 bp) from Bali cattle DNA samples

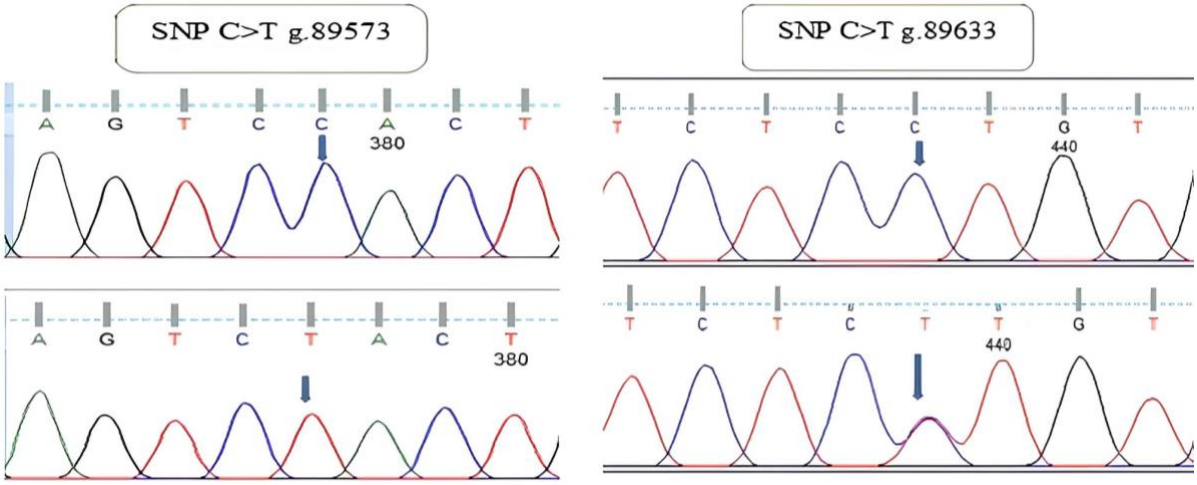

Figure 2. Visualization of SNP determination by DNA sequencing method

The specific role of the *FREM2* gene in controlling muscle and fat marbling has not been thoroughly studied. Still, *FREM2* is linked to cell adhesion and intercellular signalling, which may affect tissue development. Genetic studies in livestock suggest that *FREM2* is associated with meat quality and may influence marbling (intramuscular fat) in beef, including Bali cattle. *FREM2* has been identified as a candidate gene in studies of obesity genetics and fat metabolism in mouse models, but the exact mechanisms by which it regulates fat metabolism in muscle remain unclear. The protein complex that includes *FREM2* helps maintain tissue structure and function, which can, in turn, indirectly influence fat marbling and muscle development in livestock (Delpero et al. 2022).

The discovery of two SNPs in the *FREM2* gene of Bali cattle offers important insights into the genetic factors influencing meat quality traits in this indigenous breed. The *FREM2* gene encodes a large extracellular matrix protein involved in cell adhesion, tissue morphogenesis, and maintaining the integrity of the basement membrane (Timmer et al. 2005). These biological functions are closely associated with muscle development and intramuscular fat deposition, both of which have a direct impact on meat quality.

Population genetics analysis

Bali cattle represent a crucial genetic asset for Indonesia's beef sector due to their adaptability to tropical climates, robust resilience, and reproductive efficiency. Accounting for about 26.92% of the country's beef production, this breed is prized for its effective feed conversion, high fertility rates, and early sexual maturity (Gariri et al. 2025). SNPs found in Bali cattle can be used as genetic markers to select cattle with superior traits, such as rapid growth, increased muscle thickness, and high-quality meat. With these markers, breeding programs become more focused and efficient because they enable more accurate genetic selection compared to phenotypic selection alone. This will help improve the productivity and genetic quality of Bali cattle sustainably (Shiddieqy et al. 2019).

In another study, many genes were found to influence meat quality, particularly in Bali cattle, including six SNPs and eight insertion-deletion (indel) mutations identified in the CAPN1 gene, which showed a significant effect on various meat quality parameters. Specifically, this genetic variation was not found in either the *B. indicus* or *B. taurus* species (Dairoh et al. 2024). The ADIPOQ gene, regulated by NONBTAT000850.2 and NONBTAT000849.2, exhibited an upward trend in regulation, particularly in animals with high marbling, during the experiment. Expression patterns of lncRNA-mRNA pairs differentially expressed in relation to marbling traits in Nanyang and Angus cattle (Shi et al. 2024). Another study reported seven SNPs found in the CAPN1 and CAST genes associated with meat tenderness in the longissimus thoracis and semimembranosus muscles (Lee et al. 2019).

Functional implications of mutations

The impact of non-synonymous mutations on the *FREM2* gene is related to cattle quality, particularly in terms of meat and carcass characteristics. As shown in research conducted by Pertiwi et al. (2024) there is a non-synonymous SNP at position g.89327G>A in exon 6 of the *FREM2* gene that is significantly associated with the thickness of the longissimus dorsi muscle in Bali cattle, which is a parameter of meat quality. This mutation causes an amino acid change that can affect the function of the *FREM2* protein and impact the phenotypic traits of meat quality. In contrast, the synonym of this mutation does not cause protein changes; however, its impact on gene expression and molecular regulation can still affect the phenotypic traits of meat quality.

A missense mutation results in the substitution of one amino acid for another within a protein due to a change in the codon, whereas a nonsense mutation converts a codon into a premature stop signal, leading to truncated and incomplete protein synthesis (Su and Xia 2025). A missense mutation occurs when a mistake in the DNA code results in one of the DNA base pairs being changed. A nonsense mutation involves a single nucleotide change in the DNA sequence that converts a codon encoding an amino acid into a premature stop codon. This alteration results in the early termination of protein synthesis, producing a truncated and usually nonfunctional protein (Coddington 2020). The amino acid changes that occurred in this study can be seen in Table 1.

The change in amino acids from proline to leucine in Bali cattle has the potential to affect protein structure and function, as these two amino acids have different chemical characteristics. Proline is recognized for its distinctive ring structure, which contributes to protein stability and flexibility, while leucine is a branched-chain amino acid that influences protein synthesis and regulates muscle and fat metabolism (Rehman et al. 2023). This change could alter protein synthesis processes, metabolic potential, and even physiological properties, especially if the mutation occurs at a critical location in the relevant gene.

Genotype frequency and allele frequency

Determination of allele and genotype frequencies is essential for studies in population genetics. In studying the relationship between genotype and phenotype in a population, drawing accurate conclusions about genotype frequencies is crucial because it represents the state of a population (Maruki and Lynch 2015). The analysis revealed that both SNPs were polymorphic. Specifically, SNP C>T g.89573 exhibited a T allele frequency of 0.657 and a C allele frequency of 0.325, while SNP C>T g.89633 showed a T allele frequency of 0.200 and a C allele frequency of 0. 800.

Table 1. Amino acid changes from translated *FREM2* gene mRNA sequence

Position of mutation	Original		Mutated		Type of mutation
	Codon	Amino acid	Codon	Amino acid	
C>T g.89573	CCA	Proline	CTA	Leucine	Non-synonymous
C>T g.89633	CTG	Leucine	TTG	Leucine	Synonymous

Table 2. Genotype frequency, allele frequency, Chi-Square Test (χ^2), heterozygosity, and PIC for SNPs in the *FREM2* gene of Bali cattle

SNP position	N	Genotype frequency	Allele frequency	χ^2 Test	Ho	He	PIC
C>T g.89573	20	CT = 0.450 (9)	T = 0.675	0.013*	0.450	0.439	0.343
		TT = 0.450 (9)	C = 0.325				
		CC = 0.100 (2)					
C>T g.89633		CT = 0.300 (6)	T = 0.200	38.359	0.050	0.320	0.269
		TT = 0.050 (1)	C = 0.800				
		CC = 0.650 (13)					

Note: N: Samples total number, (n): The total samples identified with the specific genotype, χ^2 Test: Significant deviation from Hardy-Weinberg equilibrium ($p<0.05$), Ho: Observed heterozygosity, He: Expected heterozygosity, PIC: Polymorphic Information Content

Table 2 presents genetic diversity metrics, including Observed heterozygosity (Ho), Expected heterozygosity (He), Polymorphism Information Content (PIC), and χ^2 test values used to assess whether genotype frequencies conform to Hardy-Weinberg Equilibrium (HWE). Observed heterozygosity (Ho) is the proportion of heterozygous genotypes in a sample, reflecting the number of individuals with different alleles at a given locus. Expected heterozygosity (He) is the theoretical value calculated from allele frequencies under the assumption of random mating and HWE. For SNP C>T g.89573, Ho (0.450) exceeds He (0.439), consistent with the χ^2 value (0.013*) at a significance level of $p<0.05$, This finding indicates that the Single-Nucleotide Polymorphism (SNP) is in Hardy-Weinberg equilibrium, a condition typically observed in stable populations with random mating. In contrast, SNP C>T g.89633 exhibits a lower Ho (0.050) compared to He (0.320), suggesting a deviation from Hardy-Weinberg equilibrium. This observation aligns with Maskur et al. (2023), who reported that observed heterozygosity lower than expected indicates deviation from Hardy-Weinberg equilibrium. Such heterozygosity deficits may result from selective pressure on specific loci or from inbreeding.

A moderate level of heterozygosity at genes such as *FREM2* is optimal in breeding programs. This level maintains sufficient genetic variation for effective selection and minimizes the genetic fragmentation associated with rare variants. In Bali cattle, achieving this balance matters because improving genetics should not mean losing traits that help them thrive in their local environment.

The Hardy-Weinberg Equilibrium (HWE) is a foundational principle in population genetics. It provides a mathematical framework for understanding genetic variation within populations. HWE is also essential for studying evolutionary processes (Gerard 2022). The principle asserts that genotype frequencies in a population remain stable across generations unless influenced by external factors that disrupt equilibrium (Sun et al. 2018). Deviations from the Hardy-Weinberg Equilibrium can suggest genotyping errors or reflect the influence of natural selection (Kwong et al. 2021).

Hereafter, the PIC value is calculated from allele frequencies and indicates the marker's ability to genetically distinguish individuals. In this study, the SNP g.89573C>T had a PIC of 0.343 and the SNP g.89633G>A had a PIC of 0.269, both of which fall into the moderate category. This indicates that these SNPs are sufficiently informative to serve as genetic markers in breeding programmes, particularly for improving the meat quality of Bali cattle through marker-based selection.

The PIC value is a measure used to assess the level of diversity (informativeness) of a genetic marker. According to Dalimunthe et al. (2020) the Polymorphic Information Content (PIC) metric is used to assess the informativeness of genetic markers derived from PCR-amplified DNA bands. PIC scores are categorized into three levels: values above 0.5 indicate highly informative markers, scores between 0.25 and 0.5 denote moderately informative markers, and scores below 0.25 reflect low informativeness.

Although the Polymorphism Information Content (PIC) values are moderate, the identified Single-Nucleotide Polymorphisms (SNPs), especially the non-synonymous g.89573C>T variant, demonstrate potential as genetic markers for meat quality traits in Bali cattle. The functional impact of the amino acid substitution, together with the observed genetic diversity within the population, indicates that this variant may be valuable for marker-assisted selection programs (Pratiwi et al. 2016). Several critical considerations must be addressed before implementing these markers in breeding programs. First, the relationship between genotype and phenotype should be confirmed through comprehensive association studies that utilize larger sample sizes and precise measurements of meat quality. Second, the functional effects of the variants require validation using experimental methods such as functional assays or gene expression analyses (Romero et al. 2024).

Implications for marker-assisted selection

Identifying genetic variants in indigenous cattle breeds such as Bali cattle holds significant implications for conservation and the development of sustainable breeding programs. The observed moderate genetic diversity at the *FREM2* locus suggests sufficient variation to support selection initiatives while preserving overall genetic diversity (Pertiwi et al. 2024). The small effective population size of many indigenous breeds increases their vulnerability to genetic drift and inbreeding depression. Effective management of breeding programs is crucial to strike a balance between genetic improvement and the preservation of genetic diversity. Employing molecular markers facilitates the optimization of mating decisions, thereby minimizing inbreeding and maximizing genetic gain (Oakley and Winn 2012).

The development of breed-specific genetic markers is particularly significant for indigenous breeds, such as Bali cattle, which often exhibit distinct responses to selection pressures compared to commercial breeds (Margawati et al. 2019). Their unique genetic backgrounds and adaptations to tropical environments can lead to different genotype-phenotype relationships than those observed in temperate breeds (Hapsari et al. 2025).

This study has some limitations that need to be mentioned. First, the sample size was small and came from only two sources. This makes it harder to detect small differences from equilibrium and may not capture the full genetic diversity of Bali cattle in Indonesia. Future studies should combine *FREM2* genotypes with detailed carcass and meat-quality records from larger and more geographically diverse Bali cattle populations. This approach will help confirm the link between the g.89573C>T variant and important economic traits.

In conclusion, this study presents the first systematic characterization of *FREM2* Single-Nucleotide Polymorphisms (SNPs) in Bali cattle (*Bos javanicus*), identifying two novel variants: g.89573C>T, a non-synonymous substitution (Pro to Leu) in exon 6, and g.89633G>A, an intronic variant in intron 7. Both variants exhibit moderate Polymorphism Information Content (PIC) values (0.343 and 0.269, respectively) and allele frequencies suitable for Marker-Assisted Selection (MAS). These SNPs provide essential baseline data for the development of race-specific MAS programmes in Bali cattle, facilitating targeted selection for improved meat quality while maintaining critical adaptive traits for small-scale livestock production systems in tropical regions. SNP assists the MAS programme in accelerating genetic gain, reducing inbreeding, and supporting the conservation of tropical Bali cattle with high accuracy (better than data using only phenotypic data). The functional variation g.89573C>T, which is in Hardy-Weinberg equilibrium ($\chi^2 = 4.62$, $p = 0.032$) and shows excess

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