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SNP Detection in *FREM2* Gene and Its Association with Carcass Quality in Bali Beef

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ABSTRACT

The *FRAS1-related extracellular matrix protein 2* (*FREM2*) gene is one of the genes that play a role in controlling marbling scores in beef cattle. This study aimed to identify SNPs in exon 6 of the *FREM2* gene and its association with carcass quality in Bali beef using ultrasonography. A total of 93 cattle were used: 55 cattle from Banjarmasin slaughterhouse, South Kalimantan, Indonesia, 28 cattle from Bali Cattle Breeding Centre in Bali Province, and 10 cattle from UPTD Kupang Regency, NTT, Indonesia. SNP of the *FREM2* gene was identified by using sequencing techniques and then genotyping by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The association of *FREM2* gene SNPs with carcass characteristics was analyzed using the General Linear Model (GLM) method using SPSS software version 22. Carcass (*longissimus dorsi* thickness; back fat thickness) and meat (intramuscular fat; marbling score) characteristics were identified non-invasively using ultrasonography images and then analyzed with Image-J NIH software. SNP at position g.89327G>A was located in exon 6 of the *FREM2* gene and did not change the amino acid (proline→proline) sequence in Bali beef. The genotyping results with PCR-RFLP technique SNP g.89327G>A *FREM2*|*Bcc*I gene has high diversity. The alleles of A and G were 0.747 and 0.253, respectively. The diversity of SNP g.89327G>A was significantly associated (p<0.05) with *longissimus dorsi* thickness (LD), while back fat thickness, intramuscular fat, and marbling score did not significantly different (p>0.05). Thus, SNP at position g.89327G>A in exon 6 of the *FREM2* gene might be used as a candidate genetic marker for carcass quality in Bali beef.

Keywords: Bali beef; carcass quality; FREM2 gene; SNP

INTRODUCTION

Bali beef (*Bos javanicus*), domesticated on the island of Bali, is the primary cattle breed in Indonesia (Bulkaini *et al.*, 2022). The advantages of Bali beef include being able to adapt to unfavorable environments and a birth rate of 75%-85% (Wawo, 2018). Bali beef is a native Indonesian beef cattle with great potential to produce quality meat compared to other local and imported cattle (Tahuk *et al.*, 2018). The Bali beef has a carcass percentage of approximately 56% (Hafid *et al.*, 2019). Carcass quality measurement parameters are dressing percentage, intramuscular fat content (IMF) or marbling fat score, loin eye area, and backfat thickness (Li *et al.*, 2013). Carcass characteristics can be used to assess the superiority of an animal. *Ribeye muscle* and *subcutaneous fat* depths are used as indicators to determine the amount of saleable meat obtained from carcasses. Carcass traits are influenced by animal age, feed management, and animal genetics (Raza *et al.*, 2018).

Slaughter weight, carcass weight, carcass components, and fat are strongly influenced by the age of the animal (Zajulie *et al.*, 2015). Fat content in carcasses increases with the animal's age but influences the fat content of the carcass (Jakaria *et al.*, 2017). Ultrasonography images have been widely used to observe the intramuscular fat of beef both quantitatively and qualitatively (Jakaria *et al.*, 2017). Determination of carcass quality and the thickness of the longissimus dorsi has been done non-invasively using ultrasound technology at the position of the 12th-13th ribs (Jakaria *et al.*, 2017). Ultrasound technique has been proven effective in measuring IMF percentage for determining carcass quality characteristics (Nogalski *et al.*, 2018). Ultrasound has a high correlation coefficient in determining carcass quality in Bali cattle, which ranges from 0.291 to 0.938 (Jakaria *et al.*, 2017).

The development of molecular technology greatly supports the progress in animal husbandry in conducting selection using DNA markers. Marker-assisted selection (MAS) is a genetic-based molecular technology that is more effective than traditional breeding systems (Moniruzzaman *et al.*, 2015). The genes reported to be responsible for controlling meat quality are diacylglycerol O-acyltransferase 1 (*DGAT1*) and calcium-activated neutral protease μ-calpain (*CAPN1*) (Li *et al.*, 2013), acetylcholine receptor subunit alpha (*CHRNA1*), isocitrate dehydrogenase 1 (*IDH*), amyotrophic lateral

sclerosis 2 (*ALS2*), Sp1 transcription factor (*SP1*), retinoic acid receptor gamma (*RARG*), collagen type IX alpha 3 (*COL9A3*), fatty acid binding protein 4 (*FABP4*), and insulin-like growth factor 1 receptor (*IGF1R*) (Lee *et al*., 2013), lactalbumin alpha (*LALBA*), H1 histone family, member N, testis-specific (*H1FNT*), Serine Protease 33 (*PRSS33*), transcription elongation factor B (*TCEB2*), FLYWCH family member 2 (*FLYWCH2*), N-alpha-acetyltransferase 60 (*NAA60*), and caspase-16 (*CASP16*) (Magalhães *et al*., 2016), Leptin (Hilmia *et al*., 2015), Calpastatin (*CAST*), Calpaim (*CAPN*), and stearoyl-CoA desaturase (*SCD*) (Jakaria *et al*., 2020), and Calpain (Dairoh *et al*., 2021).

The *FREM2* gene encodes the protein, which is a membrane protein involved in various cellular processes, including cell growth, apoptosis, and differentiation. Protein is one of the components that affect carcass characteristics and meat composition of Bali beef (Li *et al*., 2014). Meat quality is closely related to carcass traits, and meat quality can be determined based on the Korean cattle carcass grading system (BCGS) (Bedhane *et al*., 2019). Based on the results of genome-wide association study (GWAS) analysis in Hanwoo cattle, several genes were found that affect meat quality, namely NHL Repeat Containing 3 (*NHLRC3*), Proline and Serine Rich 1 (*PROSER1*), Stomatin-like protein 3 (*STOML3*), and FRAS1-related extracellular matrix protein 2 (*FREM2*) genes. Several studies reported that the *FREM2* gene has been explored in various types of livestock, namely zebu and taurine cattle (Grigoletto *et al*., 2020), pigs (Óvilo *et al*., 2022), sheep (Zhao *et al*., 2022), and Yak (Cai *et al*., 2021). Bedhane *et al.* (2019) stated that the *FREM2* gene is a candidate for meat quality in Hanwoo cattle. Furthermore, Grigoletto *et al.* (2020) also reported that the *FREM2* gene is associated with marbling fat in Zebu beef and Taurine beef. Based on this information, identification of the *FREM2* gene on carcass quality in local beef in Indonesia, especially Bali beef, needs to be done. Therefore, this study aims to identify the SNP of the *FREM2* gene and its association with carcass quality in Bali beef.

MATERIALS AND METHODS

Animal and Tissue Collection

This research procedure was approved by 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 Udayana University, Denpasar, Indonesia, with number B/184/un14.2.9/pt.01.04/2021. A total number of 93 Bali beef were used with an age range based on teeth I_2-I_4, consisting of 55 males from Banjarmasin Slaughterhouse, South Kalimantan, Indonesia, 28 cattle (16 males and 12 females) from Bali Cattle Breeding Centre in Bali Province, Indonesia, and 10 females from UPTD Kupang Regency, NTT, Indonesia. The feeding composition of the animals was based on the standard feeding requirements of 10% forage and 1% concentrated feed of body weight.

DNA Isolation and Amplification of *FREM2* Gene

Total DNA extraction was carried out using a modified Geneaid DNA Kit procedure (Geneaid Biotech Ltd., New Taipei City, Taiwan). Blood samples were taken about 300 µL in a 1.5 mL microcentrifuge tube, and 900 µL of Red Blood Cells (RBC) lysis solution was added and then homogenized. The solution mixture was allowed to stand at room temperature for 10 minutes, then centrifuged at 3,000 rpm for 5 minutes, and the supernatant was discarded. A total of 100 µL of RBC lysis and 200 µL of GB buffer were added and then homogenized with a vortex mixture. The samples were incubated at 60 °C for 10 minutes and inverted every 3 minutes. 5 µL of RNAse was added and incubated at room temperature for 5 minutes. A total of 200 µL of ethanol absolute was added, and the sample was transferred to the GD column, then centrifuged at 14,000 rpm for 5 minutes, and the 2 mL collection tube was discarded. A total of 400 µL of W1 buffer solution was added to the GD column with a new collection tube and then centrifuged at 14,000 rpm for 1 minute. The supernatant was discarded, and the GD column was centrifuged dry again. The GD column tube was transferred to a 1.5 mL microcentrifuge tube, added 100 µL pre-heated elution buffer, and allowed to stand for 3 minutes, then centrifuged 14,000 rpm for 3 minutes. Then, the quality and quantity of DNA were analyzed by spectrophotometer and electrophoresis with 1% agarose gel. DNA quality is determined based on PCR techniques to see the DNA bands that appear, while DNA quantity can be determined based on DNA concentration. According to Budiman *et al.* (2018), the ideal ratio of DNA purity level is 260/280 nm (1.80-2.20).

Amplification of the *FREM2* gene used forward primer 5′-ACG ATG GAC CAT GAA AAT GCT-3′ and reverse 5′- CGT CGT CTT TGT CGG TTT G-3′ with a fragment length of 351 bp designed by primer3 program (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and primer stat (https://www.bioinformatics.org/sms2/pcr_primer_stats.html) with ensemble access number ENSBIXG00005003071. The PCR reaction consisted of Bioline MyTaq HS Red Mix (Meridian Bioscience, Memphis, USA) 12.5 µL, nuclease-free water (NFW) 10.1 µL, forward and reverse primers 0.2 µL each (concentration 25 pm/mL), and template DNA 2 µL (concentration 66.1 ng/µL) for a total PCR reaction volume of 25 µL. PCR conditions included pre-denaturation at 95 °C for 1 min, denaturation at 95 °C for 15 sec, annealing at 58 °C for 15 sec, and extension at 72 °C for 10 sec (total cycle 35), followed by the final extension of DNA molecules, which occurs at 72 °C for 5 minutes. Then, PCR products were electrophoresed using 1.5% agarose gel.

Sequencing and Genotyping Analysis of *FREM2* Gene

SNP g.89327G>A was found in the *FREM2* gene in exon 6 in Bali beef based on amplification results at 58 °C annealing temperature (Figure 1a). Genotyping of SNP g.89327G>A using chain reaction-fragment length polymorphism (PCR-RFLP) technique with cutting enzyme *Bcc*I (5′-...CCATC(N)4*...-3′) found two genotypes, namely genotypes GA and AA (Figure 1c).

The diversity of SNP g.89327G>A of *FREM2* gene in Bali cattle is presented in Table 1.

The PCR product was sequenced using an ABI PRISM® 3730X1 Genetic Analyer BigDye® Terminator v3.1 machine (Applied Biosystems, Thermo Fisher Scientific Inc. USA) at the 1st Base Services Selangor Malaysia. SNP has been obtained from sequencing results determined by cutting enzymes that can recognize using the Nebcutter program (http://nc2.neb.com/NEBcutter2/). Determination of the genotype of the *FREM2* gene SNP obtained was carried out using the polymerase (PCR-RFLP) method (Tsukahara, 2018). PCR-RFLP technique with enzyme *Bcc*I had reaction 5 µL of the amplicon, 0.3 µL enzyme restriction, 0.7 µL of buffer enzyme, and 1 µL of nuclease-free water (NFW), which were incubated at 37 °C for 4 hours. Visualized products were performed by electrophoresis using 3% agarose gel and the fragments were visualized under UV Transilluminator (Alpha Imager; Alpha Innotech, Santa Clara, CA, USA). The identification of genotypes was figured out based on the pattern of emerging bands.

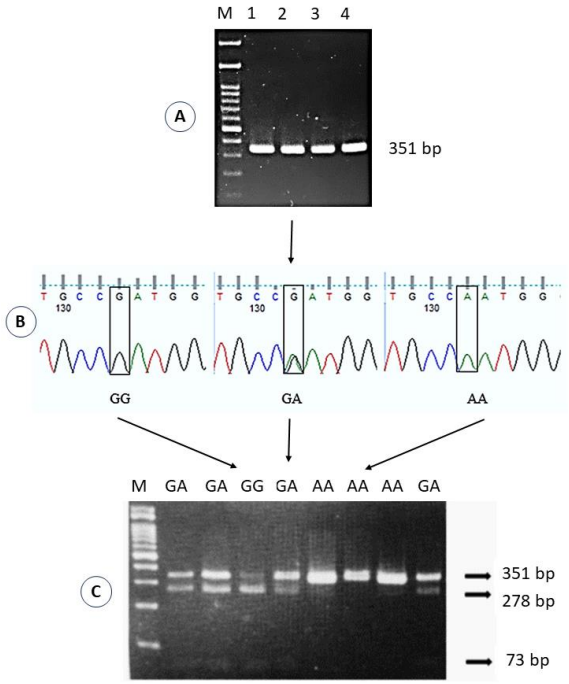

Figure 1. (a) PCR product of FREM2 gene, (b) SNP determination by sequencing, (c) genotype determination by PCR-RFLP cut with *Bcc*I restriction enzyme (CG | AT) of Bali cattle. M: 100 bp ladder.

Determination of Carcass Quality Using Ultrasound

Ultrasound data were saved in JPEG format and performed with NIH Image-J software (ImageJ, NIH, USA) (Figure 2). The determination of the marbling score (MS) was based on Meat Standards Australian (MSA) (http://www.wagyu.org.au/marbling/).

Data Analysis

SNP determination was analyzed using the Bioedit program (Hall, 1999), and SNP determination was identified using Molecular Evolutionary Genetics Analysis 7 (MEGA7) (Kumar *et al.*, 2016). PCR-RFLP data were analyzed for allele and genotype frequencies and observed heterozygosity (Ho) and expected heterozygosity (He) were calculated based on Nei & Kumar (2000). Hardy-Weinberg equilibrium (χ^2) was calculated with the PopGene 1.32 program (Yeh *et al.*, 2000). The association of *FREM2* genes with carcass quality was based on the General Linear Model (GLM) method using SPSS Software version 22 with the following mathematical model (George & Mallery, 2019):

$$Y_{ij} = \mu + G_i + S_i + e_{ij}$$

where, Y_{ij} is the response to the observed variable (carcass quality), μ is the overall mean, G_i is the effect of genotype i, Si is the effect of sex i, and e_{ij} is the error component arising in the j-th replication of genotype i.

Differences in livestock location of origin were corrected based on the following formula (Salamena & Papilaja, 2010):

Xi corrected= [$\bar{X}$ standard/$\bar{X}$ observation] x Xi observation

Where, Xi corrected is the corrected data, $\bar{X}$ standard is the mean of the standard group, $\bar{X}$ observation is the mean of the observation group, and Xi observation is the value of the ith observation.

(a) (b) (c)

Figure 2. Ultrasound imaging process in Bali cattle (a) transverse view, (b) longitudinal view, (c) the position of muscle ultrasound measurement on 12-13 ribs orientation point (BFT= back fat thickness; LD= *longissimus dorsi thickness*; IMF= intramuscular fat content, and measurement area IMF 30 mm x 30 mm.

RESULTS

SNP Detection and Diversity of *FREM2* Gene

SNP at position g.89327G>A of the *FREM2* gene has the highest frequency of A allele compared to G in both males and females that were 0.747 and 0.253, respectively, while the total frequencies of genotypes GG, GA, and AA were 0.01, 0.48. and 0.59, respectively (Table 1). The Hardy-Weinberg equilibrium test (χ^2) showed that males were unbalanced while females were balanced. However, in the total population, the g.89327G>A SNP of the *FREM2* gene in Bali cattle is unbalanced ($p<0.05$).

Association of *FREM2* Gene SNPs with Carcass Quality

The expression of *FREM2* gene SNP g.89327G>A was significantly associated ($p<0.05$) with *longissimus dorsi thickness* (LD). At the same time, back fat thickness (BFT), marbling score (MS), and intramuscular fat (IMF) did not have significant differences ($p>0.05$) (Table 2). Genotype AA had higher *longissimus dorsi thickness* (LD) compared to genotype GA.

DISCUSSION

SNP at position g.89327G>A of the *FREM2* gene is located in exon 6, which is G, change to A. This SNP has not been reported in other breeds in the world based on the *FREM2* gene reference (ENSBTAG00000017032) (https://asia.ensembl.org/). However, this SNP does not change the amino acid sequence (CCG/CCA; (proline→proline)). Extreme mutations are mutations that cause changes in amino acids (transversions) (Ali & Borah, 2021). The mutation that occurs in this case is a silent mutation where there is a change in the arrangement of nitrogenous bases that does not cause changes in the amino acids formed in protein synthesis. The CCG genetic code changes to CCA codes for the amino acid proline, so this mutation has no amino acid changes. SNP g.89327G>A was significantly associated ($p<0.05$) with *longissimus dorsi* thickness, and intramuscular fat deposition in the *longissimus* muscle influences carcass quality and acts as a palatability attribute.

Genotyping SNP at position g.89327G>A of FREM2 gene by PCR-RFLP method using *Bcc*I restriction enzyme obtained genotype GG, GA, and AA. The PCR-RFLP method has good sensitivity and is more efficient and effective for genotyping (Singh *et al.,* 2014; Hashim & Al-Shuhaib, 2019). The combination of the PCR-RFLP technique and the *FREM2* gene marker is essential to identify the association of the gene with carcass quality. PCR-RFLP results found only one fixed GG genotype (Table 1). This may be due to the negative selection that occurs in the population.

Based on the chi-square test (χ^2) ($p<0.05$), males are not in the Hardy-Weinberg equilibrium, while females are in the Hardy-Weinberg equilibrium. This happens because males have greater Ho and He values compared to females. According to Chesnokov & Artemyeva (2015), if the Ho < He indicated inbreeding, whereas if Ho > He indicated random mating in the population. The Hardy-Weinberg equilibrium in Bali cattle is due to the small sample population size. Garnier-Géré & Chikhi (2013) stated that the deviation from Hardy-Weinberg law is due to the absence of random mating, resulting in heterozygosity deficiency in large populations. Abramovs *et al.* (2020) also stated that several factors cause a population not to be in Hardy-Weinberg equilibrium, namely mutation, natural selection, non-random mating, genetic drift, and gene migration.

Genetic diversity occurs when there are two or more alleles in a population. Therefore, the allele frequency results (Table 1) in Bali cattle are categorized as polymorphic because they have more than one allele (G and A alleles). According to Bhat *et al.* (2017) and Putri *et al.* (2015), polymorphic traits have an allele frequency of more than 0.01. Furthermore, genetic diversity in a population can also be measured based on the value of heterozygosity (Ismail *et al.,* 2020).

The association of SNP g.89327G>A of *FREM2* gene with *longissimus dorsi* thickness was significant ($p<0.05$), while back fat thickness, intramuscular fat, and marbling score were not different in Bali cattle. SNP

Table 1. Diversity information of SNP at position g.89327G>A in *FREM2* gene

Sex	N	Genotype frequency			Allele frequency		Ho	He	χ^2 test
		GG	GA	AA	G	A			
Male	71	0.01 (1)	0.51 (36)	0.48 (34)	0.268	0.732	0.51	0.39	5.87**
Female	22	0 (0)	0.41 (9)	0.59 (13)	0.205	0.795	0.41	0.33	1.27ns
Total	93	0.01 (1)	0.48 (45)	0.51 (47)	0.253	0.747	0.48	0.38	7.12**

Note: N= total of samples, Ho= heterozygosity observed, He= heterozygosity of expectation, ns= not significant, **= significant at the level of 0.05, χ^2table (0.05:1) = 3.84.

Table 2. Association of *FREM2* gene with carcass quality in Bali beef

Sex	Genotype	N	Carcass characteristics		Meat characteristics	
			LD (mm)	BFT (mm)	MS	IMF (%)
Male	GA	36	44.72±5.45 [b]	3.14±0.87	1.23±0.83	2.16±1.39
	AA	34	47.72±6.34 [a]	3.53±0.94	1.61±0.92	2.63±1.81
Female	GA	9	46.03±4.47	3.61±0.52	1.31±0.76	2.46±1.49
	AA	13	47.68±6.60	3.37±0.85	1.15±0.37	2.06±0.84

Note: LD= *Longissimus dorsi thickness*, BFT= back fat thickness, MS= marbling score, IMF= intramuscular fat, GG genotype is not calculated. Means in the same column with different superscripts differ significantly ($p<0.05$).

g.89327G>A of *FREM* gene in Bali beef is a new SNP, causing other research information to be very limited, so the new SNP needs to be explored intensively, especially traits related to carcass quality under good husbandry management conditions. Other SNPs in the *FREM2* gene have been reported to be significantly associated with marbling scores including SNP (rs43287038) in BTA2, SNP (rs208621284) in BTA12, SNP (rs133022670) in BTA16, and SNP (rs134591476) BTA24 in Hanwoo cattle (Bedhane *et al.,* 2019). Meat quality traits are very difficult to improve with traditional selection as most indicators are assessed after several generations (Zalewska *et al.,* 2021). Genomic techniques offer enormous opportunities to improve the genetic potential of food-producing livestock and implement this potential in breeding programs through marker-assisted selection (MAS) (Abd El-Hack *et al.,* 2018).

Marbling score and intramuscular fat percentage are indicators in meat quality assessment. Intramuscular fat deposition or marbling in muscles, including *longissimus thoracis et lumborum*, *infraspinatus*, and *biceps femoris*, particularly in Hanwoo cattle and Japanese Black cattle, is one of the important traits affecting meat quality and palatability attributes (Park *et al.,* 2018). The level of tenderness of meat is influenced by sensory quality, namely the marbling fat content (Wang *et al.,* 2016). Increasing intramuscular fat content was positively correlated with increasing marbling scores. Intramuscular fat is a factor that strongly influences consumer perception of meat quality regarding flavor and sensory attributes (Hunt *et al.,* 2016).

CONCLUSION

The discovery of a new SNP at position g.89327G>A of the *FREM2* gene found three genotypes, including GA, GG, and AA in Bali beef. The diversity of SNP was significantly associated with longissimus dorsi thickness (LD). This SNP might be used as a candidate gene for carcass quality in Bali beef. However, this study needs to prove in a large population to confirm the role of the *FREM2* gene on carcass quality traits.

CONFLICT OF INTEREST

Jakaria serves as an editor of the Tropical Animal Science Journal but has no role in the decision to publish this article. We also certify no conflicts of interest with any financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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