BIODIVERSITAS
Volume 24, Number 11, November 2023
Pages: 6310-6315

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d241155

Single Nucleotide Polymorphism R25C and R25H on Leptin gene and their association with body weight and measurements of Pasundan cattle

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Manuscript received: 27 September 2023. Revision accepted: 29 November 2023.

Abstract. *Hilmia N, Ramdani D, Hidayat R, Widyastuti R, Hernaman I, Dudi, Edianingsih P, Arifin J. 2023. Single Nucleotide Polymorphism R25C and R25H on Leptin gene and their association with body weight and measurements of Pasundan cattle. Biodiversitas 24: 6310-6315.* The Leptin gene is a potential candidate for genetic selection in livestock. Nonsynonimous Single Nucleotide Polymorphisms (SNP) Arg25Cys/R25C in Leptin gene associated with fat deposition mainly. This study aimed to identify polymorphism of Leptin Gene based on SNPs of Pasundan cattle and their association with body weight and measurements. This research used 64 DNA samples along with the corresponding body weight and measurement data from Pasundan cattle. About 9 and 7 DNA samples from Bali and Ongole cross (PO) cattle respectively, were also collected as comparisons. The DNA along 620 bp was multiplied by PCR. The SNPs in the Leptin gene on exon two were identified, followed by direct sequencing of the PCR product. The sequencing results were analyzed by BioEdit and Molecular Evolutionary Genetic Analysis (MEGA) 4.0. The General Linear Model analyzed the association between SNPs and body weight and measurements. The results showed there were two nonsynonymous SNP in the Leptin gene on exon two, i.e. g.1047C>T/Arg25Cys/R25C (T allele) that changed amino acid Arginine to Cysteine and g.1048G>A/Arg25His/R25H (A allele) which changed Arginine to Histidine. The frequency of the C allele in the Leptin gene on Pasundan cattle (0.54) was higher than mutation alleles, i.e. T (0.29) and A allele (0.17). There were six genotypes: CC (0.266), CT (0.390), CA (0.156), TT, TA, and AA 0.063 respectively. The Leptin gene mutation at the 25th position of amino acids, which changed Arginin to Cystein or Histidin (R25C and R25H), did not affect body weight and measurement in Pasundan Cattle. Therefore, nonsynonymous SNP R25C and R25H could not be used as marker genetic selection in Pasundan cattle.

Keywords: Altering amino acids, mutation, nonsynonymous SNP

INTRODUCTION

Pasundan cattle is an indigenous cattle from West Java province established in 2014 based on the Decree of the Minister of Agriculture Number 1051/Kpts/SR.120/10/2014 (Kepmentan RI 2014). Despite being raised in extreme environmental conditions with limited feed quality, this breed has been reported to have superior reproductive performance and resistance to parasitic diseases compared to others (Widyastuti et al. 2021). Furthermore, Pasundan cattle is an essential local genetic resource, and understanding its genetic capacity is important to enhance productivity to meet beef demand, specifically in West Java. At present, the genetic capability of livestock can be explored further using molecular genetic technology. This technology is reliable in identifying livestock genetic traits that can be used as a Marked Assisted Selection to support selection effectiveness.

Several studies identified the role of hormones in physiological processes, including Leptin the product of an obese gene. As an obese hormone, Leptin hormonal system plays a crucial role in evolution by regulating the stability of fat tissue mass, safeguarding individuals from the dangers linked to being underweight (like starvation and infertility) or overweight/obesity (Friedman 2019). Leptin primarily acts on the central nervous system, particularly in the hypothalamus and neuroendocrine (Kim and Kim 2021), contributing to energy metabolism, regulation of body weight, feed intake, reproduction, immune system functions and bone development (Sainz et al. 2015; Upadhyay et al. 2015; Friedman 2019). Foote et al. (2015) stated that neuroendocrin is an essential hormone influencing feed intake and efficiency in beef cattle. The role of this hormone in bovine significantly impacts the onset of puberty, often linked to adequate body energy stores (Fernandez et al. 2020). According to previous studies, Leptin gene affects the function and concentration of Leptin hormone. Therefore, genetic influences of Leptin gene, including point mutation on the nucleotide sequence, must be considered.

Previous studies showed the impact of Single Nucleotide Polymorphism (SNP) in exon 2 of Leptin gene, showing the contribution to fat accretion. SNP is responsible for various fat-related aspects, such as carcass fat quality, fat deposition, back fat thickness, and butter fat (Kononoff et al. 2014). Further, Heryani et al. (2019) reported in Bali cattle, there is a high association (0.805) between Leptin hormone levels and the onset of the first estrus. Reports on Leptin gene polymorphism in Indonesian local cattle were

reported in Ongole Grade and Pasundan cattle from West Java Beef Cattle Breeding Centre, and there were mutations at g.1047C>T/Arg25Cys/R25C and g.1048G>A/Arg25 Histidine/R25H. These mutations in the nucleotide sequence, particularly g.1048G>A/Arg25His/R25H were specific to these cattle breeds (Hilmia et al. 2018, 2019). Fathoni et al. (2019) stated a point mutation, namely g.1048 C>T was observed on Kebumen Ongole Grade. SNP g.1180C>T was reported in Sumba Ongole and crossbreed cattle, particularly SNP g.1181G>A was found in Bali cattle (Anugratama et al. 2020). Further, Kuswati et al. (2022) found SNP g.73T>C in Madura cattle. Several studies showed that mutations in Leptin gene affect its expression by changing the concentration and function of Leptin hormone in body. Furthermore, point mutations in the nucleotide sequence C1047T/Arg25Cys/R25C, which cause a change in the amino acid coding from Arginine to Cysteine, affect the physiological function of the hormone in metabolic processes (Fortes et al. 2009).

Body weight has been reported to have a positive association with body measurement and body frame, influenced by bone and muscle development. The effect of the hormone Leptin on bone formation has also been closely linked to body measurement. Driessler et al. (2010) suggested that Leptin receptors can be found in mature primary osteoblasts and chondrocytes, indicating the presence of a direct effect on bone growth and metabolism. Tsuji et al. (2010) reported that the Leptin hormone influences bone growth by activating fibroblast growth factor 23 (FGF-23). Jomane et al. (2015) reported that Leptin gene polymorphism on SNP rs29004508 had a significant association with some growth and carcass traits in Japanese Black cattle. The study of Kuswati et al. (2022) on Madura cattle revealed a significant effect of SNP g.73 T>C on body weight, body length, and chest girth, with the TC genotype having a higher value of the parameter.

Based on results, polymorphism based on SNP R25C and R25H and the association with body weight and measurements in Pasundan cattle has not been identified. Therefore, this study aimed to determine the polymorphism of Leptin genes based on SNP and the association with body weight and measurements in Pasundan cattle.

MATERIALS AND METHODS

Materials

A total of 64 Pasundan cattle were sampled from the Tambaksari and Cijulang districts, which have the highest populations in Ciamis District, West Java. The samples were taken by purposive sampling based on phenotypic Pasundan cattle criteria from several groups of farmers. Age of the animals was more than 2.5 years, with rearing being carried out using extensive farming. They are kept in cage at night and grazing in the morning. The procedures comprised comparing 9 samples of Bali cattle and 7 samples of Ongole Grade (PO). The DNA samples were isolated from blood which taken from vena cociigea using steril venoject vacutainer with EDTA to protect coagulation. The DNA was extracted by modified phenol-chloroform method.

Methods

The sequence target of the Leptin gene along 620 bp was amplified through Polymerase Chain Reaction (PCR). Each PCR reaction contained a buffer, dNTP, primer, taq polymerase, template DNA, and dH_20. The Leptin sequence target was amplified using forward primer 5'CTCACTGCTGCGTGGTCTAC3'; and reverse primer 3'GCACTAGGATTCCGGTCTGG5' covering a part of intron 1, exon 2, and a part of intron 2. The primer was designed by Primer Basic Local Alignment Search Tool (BLAST) from NCBI. The initial denaturation was performed at 95ºC for 5 minutes, followed by 33 cycles of denaturizing at 95ºC for 45 seconds, annealing at 58ºC for 1 minute, extension at 72°C for 1 minute, and polymerase at 72°C for 5 minutes. SNPs was identified from the direct sequencing result of PCR product, which was analyzed using Bioedit and MEGA 4 program. The Basic Local Alignment Search Tool (BLAST) was used to identify similarity (homology) with gene data in GenBank (www.ncbi.nhl.nih.gov./ BLAST). Furthermore, genotyping was conducted based on SNP R25C and R25H. There were 5 genotypes e.i CC, CT, CA, TT, AA and TA. Body weight (BW) was calculated by Winter formula, body length (BL) and wither height (WH) were measured using caliper and chest girth was measured by measuring tape. The association between SNPs on Leptin gene exon 2 (genotype) as independent variable, with BW, BL, WH, CG as dependent variable was analyzed with the General Linear Model.

RESULTS AND DISCUSSION

Identification of SNPs R25C and R25H on *Leptin* Gene

The Leptin gene target through length 620 bp of nucleotide including 1st intron, 2nd exon, and a part of 2nd intron can be multiplied successfully by PCR using a specific primer, as shown in Figure 1.

Point mutation as SNPs on exon 2 of the Leptin gene was identified based on direct sequencing results and was aligned by bioedit and MEGA4 program as presented in Figure 2.

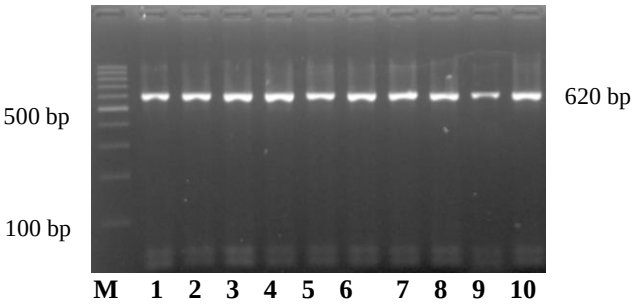

Figure 1. The sequence target of the Leptin gene (620 bp). M: Marker 100 bp; 1-10: number of individuals

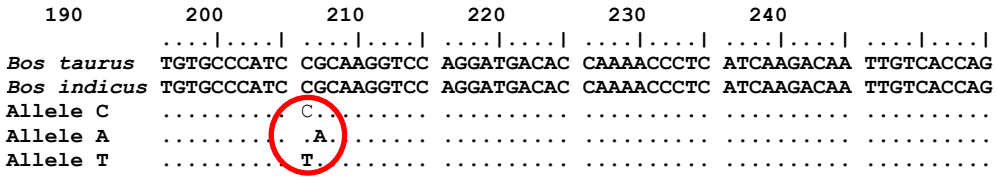

Figure 2. Alignment result of Leptin gene based on SNPs (Arg25Cys/R25C/g1047C>T/g.1180C>T) and (Arg25His/R25H/g.1048 G>A/g.1181G>A) (access number EU313203.1 and U50365.1)

Figure 2 showed that in the present study was found two nonsynonymous mutations, namely Arg25Cy/R25C/ g.1047C>T/g.1180C>T and Arg25His/R25H/ g.1048G>A/ g1181G>A. The Single Nucleotide Polymorphisms (SNPs) at exon 2 of the Leptin gene were a nonsynonymous mutation. There was a nucleotide substitution from cytosine to thymine (g.1047C>T/g.1180C>T) that changes the encoding of amino acids from Arginine (R) to Cysteine (C) (Arg25Cys/R25C) (access number NCBI EU313203.1 and U50365.1). Another nucleotide mutation in Leptin sequence was SNP Arg25His/R25H. There was nucleotide substitution from Guanine to Adenine (g.1048G>A/g1181G>A) (access number NCBI. EU313203.1 and U50365.1) that altered the encoding of amino acids from Arginine (R) to Histidine (H). Missense mutation SNP R25C on exon 2 of the Leptin gene may change the function of protein molecules, thereby altering the biological function of the gene. This was supported by cattle with homozygote SNP R25C (TT), showing high expression of Leptin mRNA. Further change of Arginine to Cysteine caused uncoupling of this nitrogen base in protein molecules. This alteration also disrupted the disulfide bonding stability, which was very important in biological function. The presence of cysteine in the A helix of Leptin molecule could impair binding to the receptors. The presence of uncoupled cysteine in protein molecules could disrupt the stability of disulfide bonding, which was crucial in biological functions (Buchanan et al. 2002)

The new nonsynonymous mutation R25H that changed the amino acid Arginine to Histidine was found in this study. This was in line with other Indonesian local cattle, such as Ongole Grade cattle. (Hilmia et al. 2018) and Bali cattle (Hilmia et al. 2019; Anugratama et al. 2020). However, it must be further studied to explore its genetic effect on Indonesian local cattle performance. The missense mutation was assumed to have the ability to alter Leptin function as an obese hormone. As an obese hormone, Leptin regulates feed intake and energy expenditure, but if there are nonsynonymous mutations which could alter amino acids, may change their function. Mutation at R25C (T allele) as stated by Fortes et al. (2009) added extra cysteine to proteins, causing the loss of protein function and showing its nature as causative. Histidine is one of the essential amino acids required for growth, blood cell production, tissue repair, and shielding tissues from damage caused by radiation and heavy metals (Moro et al. 2020). This amino acid protected nerve cells following the maintenance of myelin sheaths, and it was metabolized to the neurotransmitter histamine, essential in raising immunity, enhancing sexual function, and maintaining gastric secretion (PubChem 2018). Furthermore, Liao et al. (2013) stated histidine was the most versatile among 20 amino acids in protein architecture and bioactivity. According to previous studies, it had a unique structure that facilitated the roles in molecular interactions.

Woronuk et al. (2012) reported SNP R25C had significant positive associations with body weight and back fat. TT genotypes had higher back fat than those of CT and CC genotypes. Further, the study of Kononoff et al. (2014) revealed that increasing the T allele based on SNP R25C was significantly associated with dry matter intake, 12th Rib fat, and empty body fat. Foote et al. (2015) reported Leptin hormone concentration levels had a significant association (P<0.01), with yield grade, marbling score and 12th-rib fat thickness. The study of Leptin gene polymorphisms based on SNP R25C in Japanese black cattle reported a significant effect on C18:0 and C14:1 and suggested that SNP R25C was a potential marker for genetic improvement (Kawaguchi et al. 2016). Another study by Kononoff et al. (2017) revealed based on SNP R25C, steers with TT genotype tended to have higher dry matter intake, empty body fat, hot carcass, and carcass trait (percentage of intramuscular fat/IMF and 12th-rib fat). According to a report on commercial cattle and heifers, increasing T allele frequency tended to produce characteristic improvement in heifers (Bhowmik et al. 2019). Mutation on Leptin gene, specifically on SNP 25th amino acids coding ((Arg25Cys/R25C/g1047C>T/g.1180C>T) and (Arg25His/ R25H/g.1048 G>A/g.1181G>A) must be studied further in Indonesian local cattle, due to the essential function of Leptin hormone as a regulator of feed intake, energy expenditure, fat deposition, reproduction traits, and other physiological processes.

Based on those SNPs (R25C and R25H), each breed's alleles and genotype frequencies are presented in Table 1. As shown in Table 1, the frequency of the C allele in Pasundan and Ongole Grade cattle was higher compared to the T and A alleles. However, in Bali cattle, A allele was higher compared to C and T alleles. The results were in line with previous studies, where the frequency of C allele was higher than T allele. Fortes et al. (2009) reported that there was a low frequency of T allele, TT genotype was not found in Nelore (*Bos indicus*) cattle, while a low frequency of TT genotype (19%) was found in crossbreed species (Nelore X *Bos taurus*). The study of Kaygisiz et al. (2011) reported in 2 breeds of Turkey cattle, namely Anatolian Black cattle, where C allele frequency (0.52±0.05) was higher than T allele (0.48±0.05), as well as East Anatolian Red, with higher T allele (0.54±0.06) than C allele (0.46±0.06).

Table 1. Allele and genotype frequencies of Leptin gene based on SNPs R25C and R25H in Pasundan cattle

Breed	n	Allele frequency			Genotype frequency					
		C	T	A	CC	CT	CA	TT	TA	AA
Pasundan	64	0.540	0.29	0.170	0.266	0.390	0.156	0.063	0.063	0.063
Bali	9	0.250	0.071	0.679	0.055	0.167	0.222	0	0	0.556
Ongole Grade	7	0.857	0.071	0.071	0.714	0.143	0.143	0	0	0

Table 2. The effect of genotypes based on SNPs in the Leptin gene on body weight, body length, wither height, and chest girth in Pasundan cattle

Parameter	n	Genotype						P value
		CC n = 17	CT n = 25	CA n = 10	TT n = 4	AA n=4	TA n=4	
BW (kg)	64	264.82±37.9	263.98 ±37.3	254.84±27.6	257.78±30.0	281.60±16.6	268.00±19.9	0.85
BL (cm)	64	123.29±7.92	122.94±5.77	122.50±7.37	120.63±2.81	126.38±5.25	122.375±5.34	0.88
WH (cm)	64	114.88±3.26	114.92±4.80	115.20±3.82	112.75±4.03	115.25±3.30	114.00±3.16	0.93
CG (cm)	64	151.91±7.11	151.86±8.23	149.70±4.97	151.75±8.02	155.13±2.59	153.75±3.50	0.84

Note: BW: body weight; BL: body length; WH: wither height; CG: chest girth

Furthermore, the study of Kawaguchi et al. (2016) showed in Japanese black cattle, the frequency of RR, RC, and CC genotypes were 0.655, 0.32, and 0.025 respectively. The RR, RC and CC genotypes in Japanese black cattle were based on same mutation with this study ((Arg25Cys/ R25C/g1047C>T/g.1180C>T). The study by Fathoni et al. (2019) in Kebumen Ongole Grade indicated 0.885 of C allele and 0.115 T, respectively. Fernandez et al. (2020) in Nellore cattle found the frequency of C allele (0.82) was higher than T allele (0.12). Kuswati et al. (2022) showed among Madura cattle, C and T alleles had the same frequency of 0.5. The SNP g.1048G>A/g1181G>A/Arg25His/ R25H, A allele in Bali cattle was higher compared to those of Pasundan and Ongole Grade cattle. Furthermore, it was believed that Bali cattle were a domesticated form of wild banteng (*Bos banteng*) inhabiting the forests of Indonesia. A allele was not found in *Bos taurus* or *Bos indicus*, based on NCBI gen Bank data. This showed that A allele was considered to be specific to Indonesian local cattle (Hilmia et al. 2018). This result is in accordance with Anugratama et al. (2020), which found SNP g.1048G>A/g1181G>A /Arg25His/R25H in Bali Cattle only.

Several studies reported that there were significant associations between Leptin gene polymorphism based on SNP (Arg25Cys/R25C) with body weight (Waronouk et al. 2012), carcass grade fat, carcass yield grade, and lean meat yield (Buchanan et al. 2002; Kononoff et al. 2017), fatty acid (Kawaguchi et al. 2016), body measurements (Fathoni et al. 2019; Kuswati et al. 2022).

The association of Leptin gene polymorphisms with body weight, body length, wither height and chest girth

Body weight and body measurement were the main parameters of livestock productivity due to the association with bone, muscle, and fat growth, which contributed to daily gain as an economic value of beef farming. The effect of SNPs on Leptin gene on body weight and body measurement is presented in Table 2.

Missense mutations that altered amino acids coding in the formation of Leptin hormone could change the quantity and function of this hormone in body physiological processes, such as energy metabolism and bone formation. Altering the level of Leptin may also impact the formation of body skeleton, muscle development, and fat deposition, thereby affecting body weight and measurements. This study showed that there was no significant association between SNP R25C and R25H with body weight in Pasundan cattle. The results were consistent with several reports where polymorphisms in Leptin gene due to mutations in R25C/1180C>T /1047C>T did not affect body weight (Kononoff et al. 2014). Furthermore, the study by Hilmia et al. (2019) reported that the polymorphisms of the Leptin gene due to the R25C/1180C>T /1047C>T and R25H/1048G>A mutations in Bali cattle did not affect birth weight, weaning weight and average daily gain until weaning age. Kononoff et al. (2014) stated differences in genotype based on the Arg25Cys/ R25C SNP in the Leptin exon 2 gene did not significantly affect initial and final body weight. Based on these results, it is assumed that growth was a quantitative trait influenced by several genes, serving as additives. Furthermore, the study of Putra et al. (2019) suggested that polymorphisms in the bGHR/Alu1 g.3338A>G gene influenced body weight and measurement of Pasundan cattle. This shows that bGHR influences growth as a group of growth genes.

The size or height of the body frame in the chest, spine, and front legs contributed to the width of the height. Moreover, the height was supported by muscle and fat growth around the back and chest. In terms of fade size of Pasundan cattle, the genotypes were grouped based on mutations R25C and R25H, as shown in Table 2. The results showed that the difference in genotypes based on the 2 SNP mutations R25C and R25H in Leptin gene did not significantly ($P \geq 0.05$) affect the wither height of Pasundan cattle. This study showed that point mutations in Leptin gene that altered the amino acid coding for Arginine

to Cysteine or Histidine did not contribute to the wither height of Pasundan cattle. Similar results were obtained in previous studies, where the diversity of Leptin gene based on SNP 1180 C>T, which converted the amino acid Arginine to Cysteine, did not affect the wither height of Kebumen Ongole Grade cattle, both at weaning age and 1 year of age (Fathoni et al. 2019).

Body frame influenced body length, specifically the ribs of the sternum (thorax) and the top of the front and hind legs. It is due to body length was measured from the point of shoulder to the point of the rump. Furthermore, the formation of muscle tissue in the form of multiplication (hypertrophy) and enlargement (hyperplasia) in the chest cavity and upper legs, as well as the deposition of fat between the muscles (intermuscular) and subcutaneous fat, also had a significant contribution. The effect of SNPs on the Leptin gene on body length is presented in Table 2, with insignificant results (P>0.05). The results were in line with previous studies by Fathoni et al. (2019) where g.1180C>T mutation of Leptin gene in Kebumen Ongole Grade did not affect body length at birth, weaning age, and 1 year of age.

The effect of Leptin gene polymorphisms on the chest girth showed that genotype diversity had no significant impact (P≥0.05) on the samples. SNP R25C and R25H, which altered amino acid coding, did not significantly affect the chest girth. These results were not different from the study by Fathoni et al. (2019), that mutations in Leptin gene based on SNP g.1180C>T did not affect body measurement, including chest girth at birth and 1 year of age, but a significant effect was observed at the weaning age of Kebumen Ongole Grade cattle. Silva et al. (2014) reported mutation g.1457A>G in Nellore cattle had no significant influence on body weight, average daily gain, and carcass traits.

Body weight and measurement were quantitative traits affected by several pairs of genes, and the environment strongly influenced the expression. The results showed that there were no significant effects of mutations in Leptin gene on body measurement. Furthermore, it was suspected that skeleton, muscle, and fat deposition, were quantitative traits influenced by many pairs of genes, along with growth. Body measurement, closely related to growth, was controlled by polygenes, which contributed to the development of body skeleton, muscle proliferation, hypertrophy, and fat formation. Ferron et al. (2014) suggest that Leptin influenced and regulated osteocalcin, which regulated bone metabolism, insulin sensitivity, and energy expenditure. Furthermore, muscle cell proliferation and hypertrophy were influenced by genes related to growth, including growth hormone (GH), insulin-like growth factor (IGF), growth hormone receptor (GHR), myostatin, Pituitary Transcription Factor 1 (Pit1), and bGHR genes. Robinson et al. (2014) stated that in some cattle, there was a mutation in the myostatin gene, which caused muscle hyperplasia (increase in cell number), and affected the value of muscle growth. Furthermore, research by Altiner et al. (2017) stated that metabolism and fat deposition were very complex, comprising several hormones, such as Leptin, ghrelin, adiponectin, and insulin. Based on the results, body weight and measurement, related to growth, were influenced by several hormones coded by other genes, apart from Leptin. The research by Putra et al. (2019) suggest that mutations in the bovine growth hormone receptor gene at position g.3338A>G affected body length, shoulder height, chest girth, and body weight of Pasundan cattle. Furthermore, Gaina and Amalo (2022) found two SNPs on Myostatin gene (c.424 G>A, and c.467 G>C) and there were significant association with wither height, heart girth, and hip height (p<0.05) but no association with body weight or length.

The genetic polymorphism studies due to mutations in several genes did not significantly affect body measurement of cattle. Saputra et al. (2020) noted that genetic diversity based on mutations in the MYF5 g.643G>A gene did not affect body measurement in Bali cattle. The research of Arnim et al. (2018) revealed that variations in the Growth Hormone (GH), Insulin-like Growth Factor1 Receptor (IGF1R), and Pituitary Transcription Factor 1 (Pit1) had no influence, but mutations in the IGFR gene affected wither height in Pesisir cattle. Furthermore, Turner et al. (2013) stated that bone tissue replacement in mice lacking functional Leptin receptors (db/db) increased bone mass without affecting energy homeostasis. This showed that some of the effects of Leptin on bone metabolism could be peripheral rather than central. Further, Kim and Kim (2021) stated the multifunction and complex action of Leptin hormone could modulate numerous signaling pathways that intensified the potential of an adverse reaction. Based on the results, body weight and measurements were suspected to be quantitative traits influenced by polygene.

In conclusion, Leptin genes in Pasundan cattle were polymorphic. there were two nonsynonymous mutation/ SNPs Arg25Cys/R25C and Arg25His/R25H. Based on those SNPs, there were three alleles, C (0.54), T (0.29), and A (0.17), and there were six genotypes: CC (0.266), CT (0.390), CA (0.156), TT, TA, and AA was 0.063 respectively. The SNPs R25C and R25H did not significantly affect body weight and measurements in Pasundan cattle. Therefore, nonsynonymous SNP R25C and R25H could not be used as marker genetic selection in Pasundan cattle.

ACKNOWLEDGEMENTS

This research was supported by Padjadjaran University Lecturer Competency Research Grant (RKDU), with Contract number 1549/UN6.3.1/PT.00/2023. Therefore, we would like to thank Directorate Research and Social Services (DRPM) Universitas Padjadjaran, Sumedang, Indonesia.

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