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Diversity of partial sequence leptin gene (Exon 3) in crossbred cattle compared to GenBank database

DEVI ERMAWATI[1,*], PANJONO[2], SIGIT BINTARA[1], TELYS KURLYANA[1], TETY HARTATIK[1,*,♥]

[1]Department of Animal Breeding and Reproduction, Faculty of Animal Science, Universitas Gadjah Mada. Jl. Fauna No. 3, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia. Tel./fax.: +62-274-513363, ♥email: tety@ugm.ac.id

[2]Department of Animal Production, Faculty of Animal Science, Universitas Gadjah Mada. Jl. Fauna No. 3, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia

*) equally contributed to this work

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Abstract. *Ermawati D, Panjono, Bintara S, Kurlyana T, Hartatik T. 2022. Diversity of partial sequence leptin gene (Exon 3) in crossbred cattle compared to GenBank database. Biodiversitas 23: 5606-5612.* Genetic marker method that is often used to select cattle is Single Polymorphism Nucleotide (SNP). Single Polymorphisms Nucleotide (SNP) has been found in various candidate genes, one of which is the leptin gene. Leptin is a gene that affects reproduction and cattle weight. Therefore, this research aims to identify SNP of the leptin gene (exon 3) in Crossbred cattle by comparison with GenBank data. A sample of Crossbred cattle was taken from Klaten in Central Java, Indonesia. Afterward, DNA sequencing results were analyzed with BioEdit to identify SNP. BioEdit software was used for DNA sequencing alignment part of intron 2, exon 3 and 3'UTR. The MEGA 11.1.1 program was used to analyze and edit the genetic distance and phylogenetic tree based on the SNP leptin gene. The result shows that nine variations of SNPs were found in exon 3 and 3'UTR. The phylogenetic tree shows that five type of Crossbred cattle were divided into two clusters. Group 1 (OP76919, OP76921, OP76922) closed to *Bos taurus, Bos indicus,* and Hereford (*Bos taurus*), and and group 2 (OP76920, OP76924) close to Bali cattle (*Bos sondaicus*) and Nellore (*Bos indicus*). HindIII restriction enzyme can be recommended for genotyping Crossbred cattle using PCRRFLP, but need to shift 250 bp target sequence to the right (down stream). This research provides information on SNP in the leptin gene so that it can be used for further research on the association of the leptin gene to growth and reproduction.

Keywords: Crossbred, GenBank, leptin gene, phylogenetic, SNP

INTRODUCTION

One of the problems faced by Indonesia for the improvement of the beef cattle industry sector is the difficulty of increasing livestock population and productivity. Imported beef is relatively larger than chicken meat, lamb, and imported pork. Imported beef is accounted for 21.44% of the total value of national imports (Rouf et al. 2014). Even though Indonesia is not yet self-sufficient in beef cattle, it can be settled in various ways. Apart from doing imports, it is necessary to make efforts to increase production locally by crossing and selection (Romjali 2018).

Crossing can be done to increase productivity. It can be carried out on several types of cattle, namely Crossbred. Crossbred crossing produces heterosis that occurs in Crossbred Brahman Cross with Belgian Blue (BB) cattle as double muscle cattle to increase growth characteristics (Priyadi et al. 2017). Another crossbreed is Wagyu cattle, which comes from the original Japanese cattle with descent-produced beef and good marbling intensity, high intramuscular fat content (IMF), soft texture and juicy (Connoly et al. 2020). The selection process of cattle is often considered Single Polymorphism Nucleotide (SNP). The most commonly used SNP is the leptin gene.

Leptin assumes a significant part in fat deposition, feed intake and reproduction. In cattle, this gene is located on chromosome 4 with three exons and two introns, its end result, leptin protein, has an autocrine impact that hinders insulin-invigorated glucose take-up and lessens fat tissue adipogenesis (Amitani et al. 2013). Anugratama and Hartatik (2019) reported that there was SNP leptin gene at exon 2 in Belgian Blue and Crossbred bulls Wagyu with Brahman Cross heifers. Research by Hartatik et al. (2019) showed that four single nucleotide polymorphisms in Bligon goats were detected at positions g.758G/A, g.864C/T, g.1171G/A, and g.1454G/A. SNP in the leptin gene has been associated with characteristic fat accumulation such as back fat thickness, fat content, intramuscular and Average Daily Gain (ADG) (Perez et al. 2012). Leptin is a hormone that has a role in regulating reproduction, thermoregulation, and stress (Atasoy et al. 2012; Jecmicova et al. 2018). SNP leptin gene was reported to be associated with growth (Wang et al. 2022), carcass weight (Sedykh et al. 2020), infertility (Bhowmik et al. 2018), and reproduction (Lopez et al. 2021) in cattle. Mutations in the exon region of the leptin gene cause change in the amino acids in the region, which can affect livestock productivity. For example, the candidate leptin gene (SNP) g.92450765 G>A in *Bos indicus* cattle affected production and reproduction characteristics (Yadav et al. 2021). Mandefro et al. (2021) reported SNPs in leptin exon 2 and exon 3 regions in five Ethiopian indigenous cattle breeds vis-à-vis the Korean Hanwoo breed. The SNP,

linked to missense mutation identified on exon 2. Polymorphism trait of SNP g.820C>T associated with body weight. The genetic diversity of the leptin gene was reported to be significantly related to the quality of the carcass (Uemoto et al. 2012). SNP g.4085 A>C leptin gene in chest intramuscular sheep was significantly associated with carcass weight. Identification in the diversity of the leptin gene using Bioinformatics. Bioinformatics is a tool for the interpretation of data used in molecular identification. The program uses BioEdit, which can be used in DNA sequence alignment, amino acid change analysis, and restriction enzyme mapping analysis. Restriction enzyme usage for genotyping is an effective cost method and it is a fundamental way of how PCR-RFLP works (Cheng et al. 2016). One easy, short and correct laboratory technique for SNP genotyping is polymerase chain reaction-restrict fragment length polymorphism (PCR-RFLP). In PCR-RFLP analysis, PCR-amplified DNA is identified and endonuclease is digested in the area of SNPs sites, and the fragments generated are separated with the aid of agarose gel electrophoresis (Khallaf et al. 2017). Another program that can be used is Mega 10, which is used for phylogenetic tree reconstruction. Evolutionary relationships between species are usually represented by phylogeny trees derived from multiple sequence alignments of orthologous genes. Wood reconstruction is typically performed from multiple gene data sets (such as the core gene set) (Criscuolo 2019) to enhance the overall phylogenetic signal by reducing random errors due to the small number of characters. Phylogeny helps to organize knowledge about biodiversity, structure taxonomies, and to provide insight into events that occurred during evolution. These trees show descent from a common ancestor, and much of the strongest evidence for evolution comes from common ancestry. To fully appreciate the overwhelming evidence in support of the evolutionary theory, phylogeny needs to be understood (Scheiner et al. 2017). Previous studies (Anugratama and Hartatik 2019) reported that there was SNP leptin gene at exon 2 in Belgian Blue and Crossbred bulls Wagyu with Brahman Cross heifers. This study used blood samples from Widodo Makmur Perkasa (WMP), Klaten, Central Java, Indonesia. This study hypothesizes that SNPs are found in exon 3 of the leptin gene. This research aimed to identify a SNP of the leptin gene (exon 3) in Crossbred cattle by comparison with GenBank data found in NCBI. So that, it can be used for further research on the association of the leptin gene to growth and reproduction.

MATERIALS AND METHODS

Study area

The study aimed to identify a SNP of the leptin gene (exon 3) in Crossbred cattle by comparison with GenBank data which can be found in NCBI. The research provides information about SNP in the leptin gene so that it can be used for further research on the association of the leptin gene to growth and reproduction.

Procedures

Blood samples from sampling

Seventeen (17) samples were collected from Crossbred cattle. Crossbred cattle were obtained from the crossing of Belgian Blue bull with Brahman Cross cow and Wagyu bull with Brahman Cross cow, and each blood composition of bull was ranging from 25 to 75%. The neck of the cattle whose blood was taken was cleaned using 70% alcohol by rubbing alcohol on it. Blood samples were taken as much as 3 ml from each individual through the jugular vein using a venoject set and a vacutainer tube containing EDTA, stored in a cool box for further storage in the refrigerator at -20°C until laboratory analysis.

DNA extraction

Isolation of DNA from cattle blood using gSYNC^{TM} DNA Extraction Kit (Geneaid, Taiwan) was done at the Laboratory of Genetics and Animal Breeding, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia. A total volume of 200 µL blood samples were put in a 1.5 mL tube and added with 20 µL of proteinase K, then homogenized to be well mixed. Afterward, they were incubated for 5 minutes at 60°C. Each incubated solution was added with 200 µL of GSB buffer, then was shaken to make it homogenous. The solution was incubated for 5 minutes at 60°C and every 2 minutes, was shaken. Then 200 µL of absolute ethanol was added to the solution, and the solution was shaken. The homogenous solution was transferred to the GD column and then centrifuged for 2 minutes at 10,000 rpm. After the solution was centrifuged, the liquid was discarded and the column was replaced. Afterward, W1 buffer 400 µL was added and centrifuged for 30 seconds at 10,000 rpm. The liquid was removed and centrifuged again for 3 minutes at 10,000 rpm. After that, the liquid was discarded and the color was replaced with a 1.5 mL tube and added 200 µL of elution buffer and centrifuged for 30 seconds at a speed of 10,000 rpm. This method was compiled by Latifah et al. (2016).

DNA amplification

DNA isolation product with a total of 17 samples was then amplified by molecular amplification through a temperature change mechanism using the Polymerase Chain Reaction (PCR) method. The target of the leptin gene in the sample was amplified according to the PCR program by Kurlyana using Forward primer (Leptin_3Forward): 5'- AGCTTGGAAACATGGTGGTC-3' and Reverse primer (Leptin_3Reverse): 5'-CATGATGCTCCCTGGATTCT-3' (Kurlyana, 2022). The product size of PCR was 898 bp as part of intron 2, exon 3 and 3'UTR. The PCR step included pre-denaturation at 94°C for 1 minute, denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes.

Electrophoresis agarose gel

Before running electrophoresis, agarose gel with a concentration of 1.5% was first made, to make 1.5% agarose gel, 1.5 gram of agarose powder was weighed and dissolved in 100 mL of 1xTBE . The solution was heated in

the microwave until completely dissolved (± 3 minutes). The solution was stored at room temperature until it was not too hot (± 60°C) and add the Etbr. Agarose solution was poured evenly on the mold, then the comb was mounted on the mold to form wells in the gel and left it about 30 minutes until the gel solidified. After the gel solidified, the comb was carefully removed and the gel was stored in 1xTBE . For gels that were not used immediately, the stock could be stored in 1xTBE solution at 1.5°C. The electrophoresis process was started by mixing 1 µL ddw, 1 µL of PCR product DNA with µL of loading dye. Afterward, it was inserted into the second well and so on, while the first well was used for marker size. After all the samples were inserted into the well, the electrophoresis chamber was closed and connected to a power source. The power supply was set to 100 V for 40 minutes, then the appliance was turned on by pressing the on button. When finished, the tool was turned off (Lee et al. 2012). The results of running were observed with a UV trans-illuminator. This PCR process should produce the specific DNA bands as long as 898 bp. The DNA bands appeared to glow due to the intercalation of EtBr into nucleic acids. The results were then documented with a digital camera.

DNA sequencing

The sequencing process was carried out at LPPT UGM and PT Genetics Science to see the target sequence of Leptin gene. The sequencing method was automated Sanger sequencing, a computer reads each band of the capillary gel, in order, using fluorescence to call the identity of each terminal ddNTP. In short, a laser excites the fluorescent tags in each band, and a computer detects the resulting light emitted. Because each of the four ddNTPs is tagged with a different fluorescent label, the light emitted can be directly tied to the identity of the terminal ddNTP. The output is called a chromatogram, which shows the fluorescent peak of each nucleotide along the length of the template DNA.

Restriction enzyme mapping

SNPs (n: 17 samples) that have been detected by restriction enzymes using BioEdit, if there is the right enzyme that can cut SNP, the enzyme is used for genotyping by the PCR-RFLP method. In this technique, PCR amplicons are treated with specific fragment cuts DNA at unique palindromic sites known as a recognition sites for generating multiple DNA fragments within its different sizes. Digested amplicons are loaded, put on the gel and applied to an electric field, and various sizes of bands migrate across the gel at different distances (Hayder et al. 2019)

Data analysis and phylogenetic reconstruction

All sample sequencing results are aligned by using BioEdit software and included the sequencing results from GenBank as a comparison. The detected SNPs along the alignment sequence of individual Crossbred cattle were confirmed by chromatogram in order to get the information of genotype and mutation type. The consensus sequence of each type Crossbred cattle were used to make the construction of phylogenetic tree and genetic distance. The genetic distance of the sample and GenBank was determined using the 2-parameter kimura method, neighbor-joining CIT (NJ) tree with 1000 bootstrap analysis by Mega 11.1.1 software. Accurate estimates of genetic differences are required for research in evolutionary biology (Nishimaki and Sato 2019). Phylogenetic reconstruction analysis was determined using Mega 11.1.1 (Pramod et al. 2019) and compared to GenBank *B. indicus* (EU313203.1), *Bos taurus* (U50365.1), Hereford (NC_037331.1), Nelore (NC_032653.1), *Bos grunniens* (EU603265.1), *Bos frontalis* (EU642566.1), and the buffalo (*Bubalus bubalis*, AH 015754.2) as out of the group. The native cattle of Indonesia (three type of Bali cattle, OP748255- OP748257) and five type of Crossbred cattle (OP76919- OP76923) from this study were examined for construction of phylogenetic tree.

RESULTS AND DISCUSSION

Results

The result of the sequence is in accodance with the target (GenBank no. U50365.1 at 2835 to 3732) that include a part of intron 2, exon 3 and 3'UTR of the leptin gen (Figure 1). Multiple alignment by ClustalW in BioEdit program identified the SNP of the leptin gene in Crossbred cattle and data sequences from GenBank of B. taurus (U50365.1), B. indicus (EU313203.1), B. frontalis (EU642566.1), B. grunniens (EU603265.1), Hereford Breed (NC_037331.1, and Nelore Breed (NC_032653.1). As a result, four SNPs were identified in exon 3 regions: g.3011C>S, g.3257C>Y, g.3260T>Y,and 3272T>Y. Another five SNP was found in 3'UTR region: g.3468G>R, g.3484G>R, g.3521G>R, g.3551T>Y, and g.3661G>A (Table 1). The results in this study shows 4 SNPs were missense in CDS_S region (Exon3). Missense mutation SNPs was found on g.3011C>S, g.3257C>Y, g.3260 T>Y, g.3272 T>Y. The first SNP g.3011 C>S changes the amino acid from proline to arginine. The second SNP g.3257C>Y changes the amino acid Alanine to Valine. The third SNP g.3260T>Y changes the amino acid Leucine to Serin. The fourth SNP g.3272T>Y also a missense mutation that changes the amino acid from Leucine to Proline. (Table 2). Nine SNPs along the target sequence was include in the transition mutation (Pirimidin change to pirimidin or purin change to purin). Sequence data obtained from 17 Crossbred cattle can distinguish the sequence variation into five types. This result give the information about diversity of Crossbred cattle in Indonesia. Furthermore, the representative sequence has been registered to GenBank with the accession number OP76919, OP76920, OP76921, OP76922, and OP76923.

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2821 gagcccagga gaccagcttg gaaacatggt ggtcacgtgg gcacaagaag taagggccca
2881 gggaggatgg tgtggaagcg ggggaggaag cacctctacg ctctagggaa aggcggagtc
2941 aggggagctc tgaggagctg ccctctctcc cactgagctc ttgctctccc cttcctcctg
3001 catagcagtc cgtctcctcc aaacagaggg tcactggttt ggacttcatc cctgggctcc
3061 accctctcct gagtttgtcc aagatggacc agacattggc gatctaccaa cagatcctca
3121 ccagtctgcc ttccagaaat gtggtccaaa tatccaatga cctggagaac ctccgggacc
3181 ttctccacct gctggccgcc tccaagagct gccccttgcc gcaggtcagg gccctggaga
3241 gcttggagag cttgggcgtt gtcctggaag cttccctcta ctccaccgag gtggtggccc
3301 tgagccggct gcaggggtca ctacaggaca tgttgcggca gctggacctc agtcccgggt
3361 gctgaagcct tgaaggcctc tcttcccaaa gtccagggaa gaaacctgag cttctggctg
3421 tccacaggag aagagagcct atgtgggcat cctttatgca ggccagcggg ccatttctct
3481 ctcgctcctc tcagctgctc ttccaaaggc agaaaactgc gaggcaggaa accaaagata
3541 taaatacaga ttccacgccc accgggaagg ggggcccatc cagcaaacac tagaccggag
3601 ctgggatttt cacagcagtc ttcctccctg ttccagctcc ctctcactgc atgcttcagc
3661 gtgacctggg gtgatttcag agcctttgga ccatcaagca agattccctc tgagaatcca
3721 gggagcatca tgaaggctac agcacataca gctggatatt cccacacaac atacgatgga
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Figure 1. Sequence target of leptin gene in Crossbred beef cattle (Underline: exon 3, Yellow shadow: primer sequence of Leptin gene)

Table 1. Alignment of leptin gene in Crossbred type I, Crossbred Type II, Crossbred Type III, Crossbred Type IV, Crossbred Type V, *Bos taurus*, *Bos indicus*, *Bos frontalis*, *Bos grunniens*, Hereford (*B. taurus*), Nelore (*B. indicus*)

SNP (U50365.1)	Crossbred Type I	Crossbred Type II	Crossbred Type III	Crossbred Type IV	Crossbred Type V	Bos taurus	Bos indicus	Bos frontalis	Bos grunniens	Hereford (Bos taurus)	Nelore (Bos indicus)
3011 C>S	C	S	C	C	S	C	C	C	C	C	C
3257 C>Y	C	Y	C	C	Y	C	C	C	C	C	C
3260 T>Y	T	Y	T	Y	Y	T	Y	T	Y	Y	Y
3272 T>Y	T	Y	T	Y	Y	T	Y	T	T	T	Y
3468 G>R	G	A	G	R	R	G	G	R	G	G	G
3484 G>R	A	G	R	R	R	G	G	G	G	G	G
3521 G>R	G	A	G	G	R	G	G	G	G	G	G
3551 T>Y	T	T	T	Y	Y	T	T	T	T	Y	T
3661 G>R	G	R	R	G	S	G	R	R	G	G	G

Note: A: Adenin; C: Cytosine; G: Guanine; T: Thymin; S=C/G; Y=T/C; R=G/A

Table 2. Amino acid analysis and mutation type of leptin gene

SNP (U50365.1)	Location	Codon	Amino Acid	Mutation
3011 C>S	Exon 3	CGG	Proline	Missense, Transition
		CUG	Arginine	
3257 C>Y	Exon 3	GCG	Alanine	Missense, Transition
		GUG	Valine	
3260 T>Y	Exon 3	UUG	Leucine	Missense, Transition
		UCG	Serine	
3272 T>Y	Exon 3	CUU	Leucine	Missense, Transition
		CCU	Proline	
3468 G>R	3'UTR	GCG	-	Transition
		GCA		
3484 G>R	3'UTR	GCU	-	Transition
		ACU		
3521 G>R	3'UTR	CGA	-	Transition
		CAA		
3551 T>Y	3'UTR	AUU	-	Transition
		ACU		
3661 G>R	3'UTR	GUG	-	Transition
		AUG		

Note: Mutations that occur in CDSs can cause amino acid changes

Table 3. Restriction mapping related to 9 SNPs in leptin gene

No.	SNP		Enzyme	Recognition	Frequency	Position	Fragment size
	(U50365.1)	This study					
1	3011 C>S	g.177 C>S	BsmBI	**C**GTCTCn'nnnn_	1	184	184, 714
			Fsp3I	**C**GTCTCn'nnnn_		184	184, 714
2	3257 C>Y	g.423 C>Y	-	-	-	-	-
3	3260 T>Y	g.426 T>Y	-	-	-	-	-
4	3272 T>Y	g.437 T>Y	HindIII	A'AG**C**T_T	1	435	435, 463
5	3468 G>R	g.634 G>R	FauI	CCC**G**Cnnnn'nn_	2	58, 626	58, 598, 243
6	3484 G>R	g.650 A>R	-	-	-	-	-
7	3521 G>R	g.687 G>R	-	-	-	-	-
8	3551 T>Y	g.717 T>Y	-	-	-	-	-
9	3661 G>R	g.827 G>R	-	-	-	-	-

Note: Restriction mapping can determine the position of the enzyme restriction

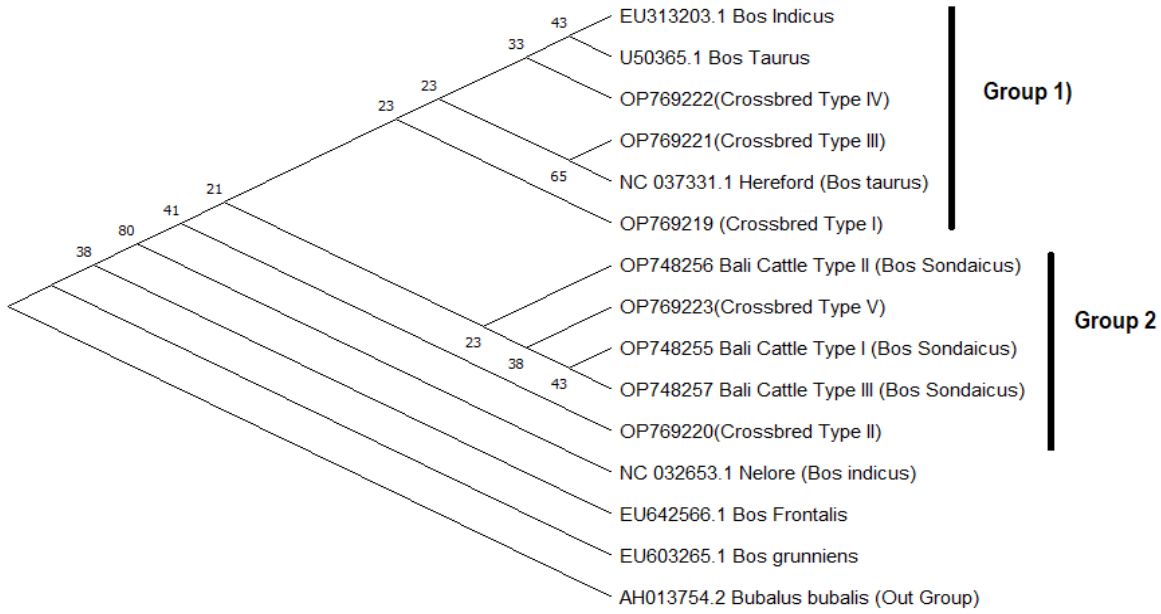

Figure 2. Phylogenetic tree based on leptin gene

Restriction mapping is the method used to analyze if restriction enzymes are associated with SNPs found useful as tools for gene marking. Cleavage is characteristic of restriction enzymes of specific sites for DNA alignment. In this study, three SNPs were identified by restriction enzymes. BsmBI (CGTCTCn'nnnn) and Fsp3I (CGTCTCn'nnnn) enzymes recognize bases at the sequence position g.184, HindIII (A'AGCT_T) enzymes recognize bases at the sequence position g.435. Enzyme FauI (CCCGCnnnn'nn_) recognizes bases at sequence position g.58 and g.626. Three enzyme recognized one site restriction (BsmBI, Fsp3I , and HindIII), and one enzyme recognized two site restriction (FauI). Based on the frequency of cutting site and the fragment size, the result can be used for genotyping Crossbred cattle by using the PCR-RFLP method. This is because the number of bands formed is not too many and the fragment size is different, so that during visualization of PCR-RFLP product, the fragment DNA in agarose gel can be seen clearly. Table 3 shows the restriction mapping related to nine SNPs in leptin gene.

A phylogenetic tree is a diagram depicting the evolutionary relationships between organisms. The phylogenetic tree was built based on the Kimura 2-parameter model using 1000 bootstrap replicas (Figure 2). As a result of Figure 2, Crossbred cattle separate into two groups: Group 1 (OP76919, OP76921, OP76922) closed to *Bos taurus*, *Bos indicus*, and Hereford (*Bos taurus*), and and group 2 (OP76920, OP76924) close to Bali cattle (*Bos sondaicus*) and Nellore (*Bos indicus*). More distance was shown in the phylogenetic tree of Bos frontalis, Bos grunniens, and Bubalus bubalis as outgroup of this study.

Discussion

Nine variations of SNPs in Leptin gene of Crossbred cattle were found in exon 3 and 3'UTR (Table 1). There are four SNPs affecting the encoded amino acids. The amino acid change was analized from BioEdit: SNP g.3011 C>S

(Pro/Arg), SNP g.3257C>Y (Ala/Val), and SNP g.3260T>Y (Leu/Ser). Genotype at the position of SNPs were confirmed by chromatogram. A four colour chromatogram showing the result of the sequencing result. Single peak of chromatogram represented the homozygote and double peak was heterozygote. SNP g.3011C>S it has two genotypes: CC and CG. SNP g.3257C>Y it has two Genotype: CC and TC. SNP g.3260 T>Y has two genotype TT and TC. SNP g.3272T>Y has two genotype: TT and TC. SNP g.3468G>R has three genotype: GG, AA, and GA. SNP g.3484G>R has three genotype: GG, AA and GA. SNP g.3521G>R has three genotype: GG, AA, and GA. SNP g.3551T>Y has two genotype: TT and TC. SNP g.3661G>R has two genotype: GG and GA. Mahrous et al. (2020) reported that several SNPs had been discovered. Among them, the T27C SNP interferes with the intronic splice muffler. The A114G, A310G, G263A, and G379A SNPs disrupt exon splicing enhancers, and the last two SNPs generate new exon splicing enhancers. Kawaguchi et al. (2017) reported a significant association between the genotype of Japanese Black and growth. Kuswati et al. (2022) reported that this SNP was significantly associated with body weight and body measurements. In addition, the leptin gene has been reported to affect fertility and production characteristics. Matumoto et al. (2022) reported that SNP c.239C>T (p.A80V) in leptin gene had significant effects on the traits related with quality grade; beef marbling standard (p: 0.0132), meat brightness (p: 0.0383), and meat firmness (p: 0.0115). Yadav et al. (2020) reported that SNP has contribution to estrus and physical condition scores. The leptin gene has been reported to affect parenting each year (D'occhio et al. 2019).

In this study, only four SNPs out of nine SNPs were identified by restriction enzymes. BsmBI (CGTCTCn'nnnn_) and Fsp3I (CGTCTCn'nnnn_) enzymes recognize bases at position g.177 and resulted two fragment (184 bp and 714 bp). The fragment size was very good, but recognition site very broadly with many unspecific nucleotide. The same condition was appeared for FauI (CCCGCnnnn'nn_) which recognized bases at position g.58 and g.626. This enzyme produced three fragment of PCR-RFLP which very clear size separately: 58 bp, 598 bp, and 243 bp. HindIII (A'AGCT_T) recognized bases at the position g.435 and produced the fragment size 435 bp and 463 bp. The fragment size was very close and difficult to separate two bands, however, the recognition site of HindIII is specific (A'AGCT_T). It can be recommended as a marker for PCR-RFLP, but need to shift 250 bp target sequence to the right (down stream). Park et al. (2015) reported that LEP/HindIII polymorphisms were significantly different for the average daily gain (ADG) in Duroc pigs ($P<0.05$). Kurlyana (2022) reported that detection of the leptin gene in Bali cattle found 3 SNP at positions g.2913 C>T, g.3260 T>C and g.3549 G>A and resulted in seven types of Bali cattle. SNP g.2913 C>T and g.3260 T>C shows the association of genotype to body size of Bali cattle.

The phylogenetic relationship of the leptin gene (Figure 2) is that Crossbred cattle type I, type III and type IV (OP769219, OP769221, and OP769222) have close relashionship with *indicus* (EU313203.1), *B. taurus* (U50365.1), and Hereford (NC_037331.1). Type II and type V of Crossbred cattle (OP769220 and OP769223) are closely related to Bali cattle (*B. sondaicus/B. javanicus javanicus*: OP748256, OP748255, OP748257) and Nelore (NC_032653.1). Very broad genetic distances was shown as *B. grunniens* (EU603266), *B. frontalis* (EU642566), and *Bubalus bubalis* (AH 015754.2) as out of the group. The results of the study by Awalia (2022) showed that the leptin gene in Friesian Holstein cattle showed four genotype base on five SNPs at positions g.3100C>T, g.3257C>T, g.3260T>C, g.3272T>C, and g.3356C>T. Thus, Crossbred cattle in this study has more diversity compared to Bali cattle (seven type) and Friesian Holstein (four type). The mutation type is the same as transition mutation. Other research on Crossbred cattle (the same sample resources with this study) is reported by Hartatik et al. (2020) that the chromosomal SRY gene in Crossbred cattle shows that there is an SNP at position g.1707 T>G (transversion mutation) and shows the contribution of the *B. taurus* gene through the paternal line. SRY gene in Bali cattle, Crossbred cattle, Bos indicus and Bos taurus has the spesific sequences (Hartatik et al, 2018). Based on the results of the sequence alignment, 9 SNPs were found in exon 3 and 3'UTR. Four out of nine SNPs indicate the missense mutations. It could affect the expression of gene and associate with economical traits and potentially be a marker-assisted selection (MAS). The results of this study provide genetic information that can be used to further investigate the relationship between leptin gene polymorphisms with animal weight and reproduction, especially on exon 3.

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