Original Research

Journal of Advanced Veterinary Research
(2025) Volume 15, Issue 6, 811-817

Screening leptin gene target for PCR-RFLP and heterosis value in crossbred cattle

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ARTICLE INFO

Recieved: 09 September 2025

Accepted: 22 October 2025

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Keywords:

Crossbred cattle, Leptin gene, Restriction enzyme, Heterosis, Growth trait.

ABSTRACT

Crossbreeding enhances the genetic potential of livestock, with Brahman Cross cattle excel in adaptability, while Belgian Blue crossbred and Galician Blonde Crossbred exhibit superior growth. This study aimed to analyze single nucleotide polymorphism (SNP) variations in the leptin gene, identify appropriate restriction enzymes, evaluate heterosis effects, and assess the association with growth traits in Brahman Cross, Belgian Blue crossbred, and Galician Blonde crossbred cattle. A total of 115 cattle were genotyped, consisting of 10 Brahman Cross, 20 Galician Blonde Crossbred, and 85 Belgian Blue crossbred. Blood samples were collected, and DNA was extracted. Amplification DNA of the leptin gene were performed using PCR method. The SNP analysis revealed that Belgian Blue crossbred has highest SNPs (17 SNPs) than Galician Blonde crossbred (9 SNPs) and Brahman Cross (10 SNPs) from a total of 25 SNPs. The BmgBI restriction enzyme was spesific for Belgian Blue Crossbred genotyping, and HindIII restriction enzyme was selected as a candidate marker for genotyping bethween Brahman Cross, Galacian Blonde crossbred, and Belgian Blue crossbred. The association of genotype and growth trait in Brahman Cross cattle was not significant. The TC genotype in Belgian Blue crossbred showed superior growth traits at birth, weaning, and one year of age. Meanwhile, the CC genotype in Galician Blonde crossbred was associated with better performance than the other genotypes. The heterosis analysis revealed that Belgian Blue crossbred displayed a higher heterosis effect compared to Galician Blonde crossbred. Single nucleotide polymorphism (SNP) variations from this study contribute to a better understanding of variation in crossbred cattle in Indonesia. These findings provide valuable insights into the genetic structure of the leptin gene and lay the foundation for marker assisted selection strategies.

Introduction

Brahman cattle, developed in the U.S. between 1854 and 1926, belong to the *Bos indicus* group and originate from several Indian breeds. To improve productivity and meat quality, they were crossbred with European cattle, creating Brahman Cross. In Indonesia, imported Brahman Cross cattle from Australia are widely reared for beef production due to their adaptability, disease resistance, and reproductive efficiency (Sutarno and Setyawan, 2016). However, their growth rate remains a limiting factor compared to *Bos taurus* breeds (Rafsanjani *et al*., 2023). To improve growth performance, crossbreeding with *Bos taurus* breeds known for their larger body size and faster growth rates has been widely practiced. Belgian Blue and Galician Blonde are two *Bos taurus* breeds extensively utilized due to their remarkable muscle growth and carcass yield (Panjono *et al*., 2022; Iglesias *et al*., 2024). The benefits gained from crossbreeding are largely attributed to heterosis. Heterosis, or hybrid vigor, occurs as a result of increased heterozygosity and produces offspring with better performance than the average of their parents, particularly in terms of growth, fertility, and environmental adaptability. Crossbreeding has been associated with improved growth performance as well as improved reproductive traits. Heterosis peaks in the F1 generation and may decline in subsequent generations if unmanaged. Overdominance (when the heterozygous allele combination performs better than either homozygous condition) and the dominance effect (when the beneficial allele masks the influence of the other) are the two main mechanisms behind heterosis (Khayatzadeh *et al*., 2018). Although heterosis provides advantages for improving development and reproductive performance, the function of specific genetic factors, such as SNPs in the leptin gene, in the Brahman cross, Belgian Blue crossbred, and Galician Blonde crossbred is still unknown. Additionally, by closely relating the mechanisms of heterosis, such as dominance and overdominance, to the crossbreeding of exotic bulls (Belgian Blue and Galician Blonde Bulls) with Brahman cross dams, it is vital to explore them. When crossbreeding cattle to improve their genetic composition, exceptional individuals must be carefully selected to guarantee that the desired traits are handed down through the generations. Farmers can progressively raise the genetic quality of their animal herd by employing marker-assisted selection. Numerous SNP markers have been identified for established and novel genes across the bovine genome (Izamin et.al., 2026). According to Diao and Lin (2020), SNPs are often employed genetic markers that modify gene expression and function, which can affect phenotypic diversity and production qualities.

Different SNP combinations in certain genes or genomic regions may cause variations in cattle's growth performance, reproduction, and other economically important traits. To find allele variations, SNP genotyping has heavily relied on restriction enzymes (Cheng *et al*., 2018). The leptin gene encodes the hormone leptin, which is released by adipocytes. It is an essential regulator of energy balance and transmits information about energy reserves to the brain (Picó *et al*., 2021). Several studies have linked differences in the leptin gene to productive traits in different cow breeds. For instance, SNP variations in the leptin gene have been connected to growth metrics including body weight and body size in Madura cattle (Kuswati *et al*., 2022) and Bali cattle (Kurlyana *et al*., 2023). Numerous other candidate genes, such as myostatin (MSTN), melanocortin 4 receptor (MC4R), and growth hormone receptor (GHR), have been extensively researched because of their significant roles in growth traits like increased muscle mass in MSTN, regulation of feed intake and body size in MC4R (Ross *et al*., 2023), and growth hormone regulation in GHR (Agung *et al*., 2024). The leptin gene encodes the leptin hormone and is a protein product of the obesity (Ob or Lep) gene. Adipose tissue-produced leptin in cattle controls feed intake and energy metabolism for grwoth by binding to receptors in the hypothalamic arcuate nucleus (ARC). Leptin regulates energy balance, which promotes somatic growth by secreting growth hormone and IGF-1, by blocking appetite-stimulating neuropeptides such NPY and activating POMC (Odle *et al*., 2018).This study assessed SNP variants in the leptin gene to identify sample types, restriction enzymes associated with these SNPs, and heterosis effects. These findings

ISSN: 2090-6277/2090-6269/

provide new insights into the genetic composition of the leptin gene and advance our understanding of genetic variation in crossbred cattle.

Materials and methods

Ethical approval granted

The Animal Ethics Commission of the Faculty of Veterinary Medicine at UGM. All procedures involving animals utilized in this investigation were approved by Gadjah Mada University, Indonesia. The approval was given under ethical number 012/EC-FKH/Eks/2023.

Samples

A total of 115 cattle were used in this investigation which contains ten Brahman Cross cattle, 20 Galician Blonde crossbred, and 85 Belgian Blue crossbred. Animals were investigated from August 2022 to December 2024, during which the data of body weigth was collected and measurement of the vital statistic data at birth, weaning, yearling, and 18 month of age were done. All cattle were raised in PT Pasir Tengah in Cianjur, West Java.

Blood sample collection and dna extraction

Blood sample was collected from the coccygeal vein of each cow after properly disinfecting the area with 70% alcohol. Approximately 3 mL of blood per animal was drawn using a syringe attached to a funnel, allowing the blood to flow naturally into the collection container tube containing EDTA which then was preserved under -20°C.

DNA extraction

DNA extraction involved breaking open the cells to release the DNA, which was subsequently purified by eliminating proteins and other cellular debris in order to get pure DNA for further analysis. The DNA from the blood sample was extracted at the Laboratory of Animal Genetics and Breeding, Faculty of Animal Science, Gadjah Mada University, Yogyakarta, Indonesia, using the gSYNCTM DNA Extraction Kit (Geneaid, Taiwan).

Amplification target sequence using polymerase chain reaction

Polymerase Chain Reaction was performed in a total volume of 25 µl, consisting of 12.5 µl of PCR Kit (KAPA BIOSYSTEMS, USA), 9.5 µl of double-distilled water (DDW), 2 µl of DNA template (50 ng), 0.5 µl of forward primer (5 µM), and 0.5 µl of reverse primer (5 µM). The PCR process used several primer pairs (leptin_1, leptin_3, leptin_4, leptin_5, leptin_9, and leptin_10). The sequence of the primer can be accesed at website https://www.ncbi.nlm.nih.gov/nuccore/PQ271568. The PCR program for all primers included an initial pre-denaturation at 94°C for 1 minute, followed by denaturation at 94°C for 1 minute, annealing at temperatures 53°C (leptin_9 and leptin_10), 54°C (leptin_4), 55°C (leptin_3 and leptin_5), 57°C (leptin_1) for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The amplification process was carried out using the Primus 25 Advanced Thermal Cycler (Germany). Following that, the PCR results were examined under a UV lamp using 1% agarose gel electrophoresis. The 3786 bp of leptin gene target sequence was constructed from overlapping of six primer sets as shown in Fig. 1.

Sequencing

The 30 µL of PCR products from each sample were sent to 1st Base Genetic Science for sequencing. The BigDye® Terminator v3.1 Cycle Sequencing Kit and the ABI PRISM 3730xl Genetic Analyser from Applied Biosystems, USA, were used in the Sanger sequencing procedure. A forward primer for the leptin gene was used for the direct sequencing process. After that, SNPs and haplotypes from three cattle groups were analyzed using Bioedit 7.5 software.

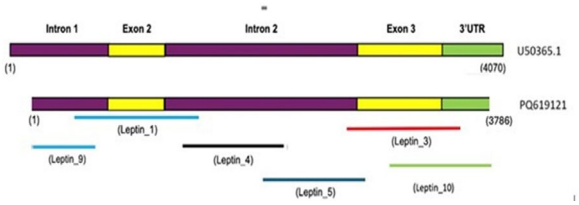

Fig. 1. Overlapping leptin gene target sequences in Brahman Cross, Galician Blonde crossbred, and Belgian Blue crossbred cattle.

Restriction enzyme mapping and genotyping by PCR-RFLP methods

Single nucleutide polymorphism identified from intron 1 to the 3′UTR (contain full coding sequence) underwent restriction enzyme mapping. The restriction enzymes were then identified by verification of sequence arround the SNPs using Bioedit 7.5 software. The restriction enzyme that can produce the clearest genotype pattern (restriction fragment) can be recommended as a marker at certain target gene. Restriction enzymes are helpful for differentiating DNA sequence variations by selectively recognizing and cutting DNA at specific target sequences, often referred to as recognition sites (Latifah *et al.*, 2018). The selected restriction enzyme (Hind III) were used for genotyping by PCR-RFLP methods.

Performance analysis and heterosis Value

Performance analysis was measured from three genotypes from PCR-RFLP results of each crossbred cattle. Body weight, body length, chest circumference, and shoulder height measurements were taken at birth, six months (weaning), 12 month (yearling), and 18 month. Heterosis value measures the improvement in performance of the F1 offspring caused by the introducing paternal genetic effect from exotic bull by artificial insemination, and it is compared to the average performance of the maternal parent (Brahman cross). Heterosis value were calculated from body weight at birth, six months (weaning), 12 month (yearling), and 18 month, with the formula as follow:

Heterosis value(%)= "(F1 value - Maternal parent value)"/"Maternal parent value" x100%

Statistical analysis

Three genotypes and growth traits were analyzed using SPSS for ANOVA for SNPs with the equation as follow:

$$Y_{ik} = \mu + \alpha_i + e_{ik}$$

Y_{ik} represents the observations data (such as body weight, body length, chest circumference, and shoulder height), µ denotes the mean of the growth trait, α_i represents the treatment effect for the genotype i, and e_{ik} stands for the random error.

Results

Amplification of target sequences was shown the product size of PCR in Fig. 2, and the representative sequence result was aligned to determine the SNP of three cattle groups (Fig. 3). Genetic diversity analysis revealed substantial variation among the crossbred cattle, with Belgian Blue crossbred has highest SNPs (17 SNPs) than Galician Blonde crossbred (9 SNPs) and Brahman Cross (10 SNPs) from a total of 25 SNPs. This variation was evidenced by the identification of distinct single nucleotide polymorphism (SNP) variants across the three cattle types. Specifically, the five unique leptin gene sequence types were identified within each

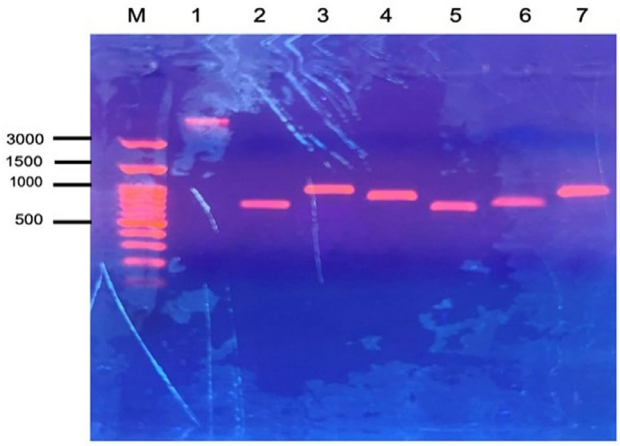

Fig. 2. Electrophoresis of six target PCR of Leptin Gene in Crossbreed cattle. M= Marker 100bp, 1 = DNA extraction; 2 = Leptin 9 (746 bp); 3= Leptin 1 (961 bp) ; 4 = Leptin 3 (898 bp) ; 5 = Leptin 4 (782 bp); 6 = Leptin 5 (871 bp); 7 = Leptin_10 (939 bp).

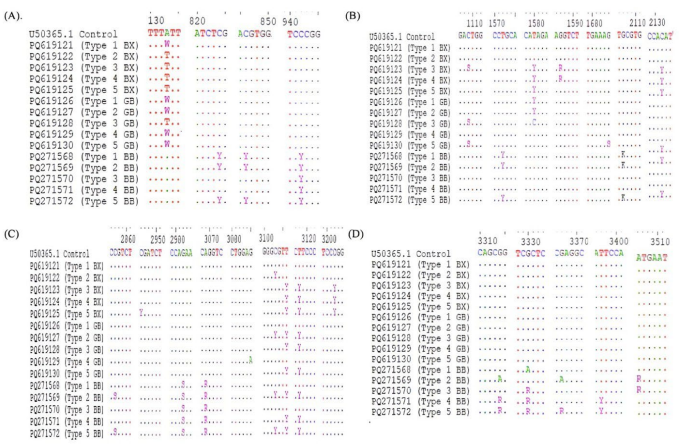

Fig. 3. Single nucleotide polymorphism of leptin gene in Crossbreed cattle. Note: (A). SNP at Intron 1, (B). SNP at Intron 2, (C). SNP at Exon 3, and (D). SNP at 3'UTR.

breed of Brahman Cross, Belgian Blue crossbred, and Galician Blonde crossbred cattle indicating a high degree of genetic differentiation (Table 1). A total fifteen sequences of leptin gene from three breeds have been submitted into GenBank: the accession numbers of Brahman Cross PQ619121–PQ619125; Galician Blonde crossbred PQ619126–PQ619130; and Belgian Blue crossbred PQ271568–PQ271572.

Based on restriction enzyme analysis, three restriction enzymes (BmgBI, XmaI, and HindIII) showed two or fewer restriction sites, indicating their potential to detect genotypes in these regions. However, the most specific recognition sequence of HindIII was the best marker for SNP g.3118C/T (Table 2) which recognized sequence 5'-A'AGCT_T-3'. The HindIII restriction enzyme detected the g.3118 T/C SNP in the leptin gene of three groups cattle, displaying different genotype patterns among the samples. Target Leptin 3 showed heterozygote CT (898 bp, 435 bp, 463 bp), homozygote TT(435 bp, 463 bp), and homozygote CC (898 bp). Target Leptin 10 showed heterozygote CT (268 bp, 271 bp, 939 bp), homozygote TT (268 bp, 671 bp), and homozygote CC (939 bp). This result indicates that HindIII is an effective restriction enzyme as a molecular marker for genotyping in the target sequence Leptin 3 and Leptin 10. While the chromatogram of SNP g.3118C/T containing sequence A_AGCT_T which recognized by HindIII restriction enzyme is shown in Fig 4A-C. The HindIII restriction enzyme was effective for genotyping by PCR-RFLP (Fig.4D). The BsmBI and Esp3I restriction enzymes were detected in three crossbred cattle, but with less specific recognition sequences, CGTCTCn'nnnn and CGTCTCn'nnnn, respectively. The restriction enzyme HpyCH4IV and BmgBI were considered appropriate for targeting the g.846 C/T SNP, showing different cutting site frequencies and generating similar fragment sizes at the first cutting site. Thus, the selection of these restriction enzymes provides a strong basis for the application of SNP for breeding programs based molecular markers in Brahman Cross, Galician Blonde crossbred, and Belgian Blue crossbred cattle.

The genotype TT, CT, and CC at SNP g.3118 T/C in Brahman Cross cattle showed no significant association with growth traits. SNP g.3118 T/C was significantly associated with several growth traits in Belgian Blue crossbred cattle up to one year of age and remained significant at later stages in Galician Blonde crossbred. However, in Belgian Blue crossbred

Table 1. Number and position of single nucleotide polymorphism in Brahman cross, Galacian Blonde crossbred, and Belgian Blue crossbred.

Breed	Haplotype (GenBank)	Number and position of single nucleotide polymorphism																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
						1	1	1	1	1	2	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3
		1	8	8	9	1	5	5	5	6	1	1	8	9	9	0	0	1	1	1	2	3	3	3	3	5
		3	2	4	4	0	7	8	8	8	0	3	5	4	8	6	8	0	0	1	0	1	3	6	9	0
		3	4	6	2	9	2	0	7	4	7	2	7	6	2	9	5	3	6	8	2	4	0	7	7	7
1.BX	I(PQ619121)	W	C	C	C	C	T	T	G	G	G	C	C	C	G	A	G	C	T	T	C	G	G	G	T	G
	II(PQ619122)	T	C	C	C	C	T	T	G	G	G	C	C	C	G	A	G	Y	T	T	C	G	G	G	T	G
	III(PQ619123)	T	C	C	C	S	T	Y	R	G	G	C	C	C	G	A	G	C	Y	Y	Y	G	G	G	T	G
	IV(PQ619124)	T	C	C	C	C	T	Y	R	G	G	Y	C	C	G	A	G	C	Y	Y	Y	G	G	G	T	G
	V(PQ619125)	T	C	C	C	C	T	Y	G	G	G	Y	C	Y	G	A	G	C	Y	Y	Y	G	G	G	T	G
2. GBC	I(PQ619126)	W	C	C	C	C	T	Y	G	G	G	Y	C	C	G	A	G	C	T	T	C	G	G	G	T	G
	II(PQ619127)	W	C	C	C	C	T	T	G	G	G	C	C	C	G	A	G	Y	Y	Y	C	G	G	G	T	G
	III(PQ619128)	T	C	C	C	S	T	C	G	G	G	C	C	C	G	A	G	C	Y	Y	C	G	G	G	T	G
	IV(PQ619129)	W	C	C	C	C	T	T	G	G	G	C	C	C	G	A	G	C	T	T	C	G	G	G	T	G
	V(PQ619130)	W	C	C	C	S	T	T	G	S	G	C	C	C	G	A	G	C	Y	Y	C	G	G	G	T	G
2.BBC	I(PQ271568)	A	Y	Y	Y	C	Y	T	G	G	K	Y	C	C	S	R	G	C	T	T	C	G	A	G	T	G
	II(PQ271569)	A	Y	Y	Y	C	Y	T	G	G	K	Y	S	C	S	R	G	Y	Y	Y	C	A	G	A	T	R
	III(PQ271570)	A	C	C	Y	C	T	T	G	G	G	C	C	C	S	R	G	C	T	T	C	G	R	G	T	R
	IV(PQ271571)	A	C	C	Y	C	T	T	G	G	G	C	C	C	S	R	G	C	Y	Y	C	R	R	G	Y	G
	V(PQ271572)	A	Y	Y	Y	C	Y	T	G	G	K	Y	S	C	S	R	G	C	Y	Y	C	R	R	R	Y	G

BX: Brahman cross; GBC: Galacian Blonde crossbred; BBC: Belgian Blue crossbred; A: Adenine; C: Cytocine; G: Guanine; T: Thymine; W: A or T (Weak); Y: C or T (pYrimidine); S: C or G (Strong); R: A or G (aRginine); K: G or T (Keto).

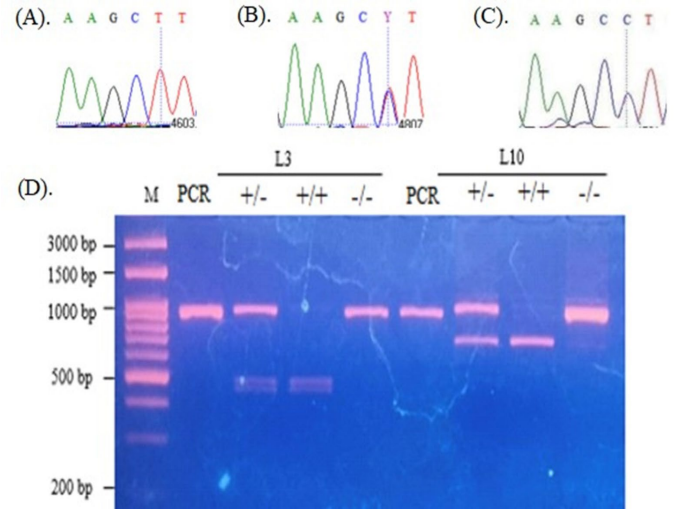

Fig. 4. Sequencing chromatogram of the leptin gene which recognized by HindIII restriction enzyme. (A) homozygote +/+(TT), (B) heterozygote +/-(TC), (C) homozygote -/-(CC) of crossbreed cattle, and (D). PCR-RFLP with HindIII Enzyme at leptin 3 (L3, 898 bp) and leptin 10 (L10, 939 bp) target gene.

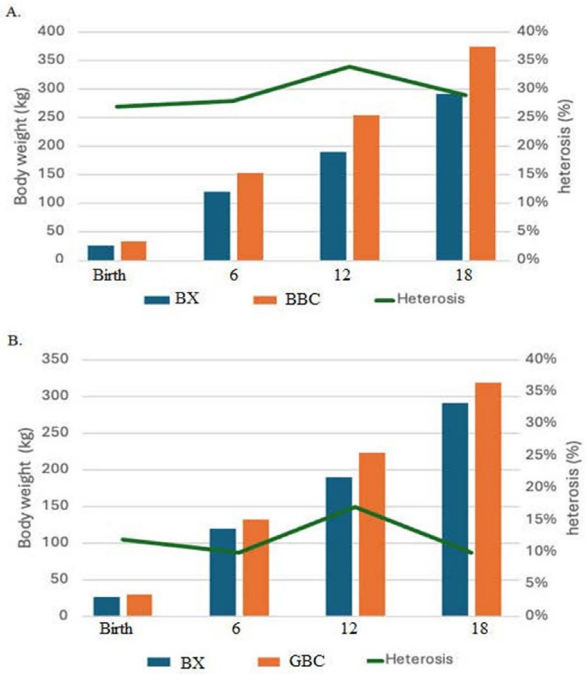

Fig. 5. Heterosis value at birth, 6, 12 and 18 month (A) Heterosis value in Belgian Blue crossbred, (B) Heterosis value in Galician Blonde crossbred.

cattle, this SNP was significantly associated with various growth parameters. Individuals with the TC genotype in Belgian Blue crossbred exhibited significantly greater body weight, body length, chest girth, and wither height compared to those with TT and CC genotypes at birth, weaning (6 months), and one year of age. Nevertheless, these associations were no longer significant at 1.5 years of age. In addition, research findings also revealed that SNP g.3118 T/C was significantly correlated with body weight, body length, chest width, and height at both weaning and 1.5 years of age in Galician Blonde crossbred cattle (Table 3). Belgian Blue crossbred cattle showed body weight at various growth stages (birth, weaning, yearling, and 1.5 years) higher than Brahman Cross but lower than pure Belgian Blue. The heterosis values were 27.52%, 28.04%, 33.74%, and 28.68% , respectively. In Galician Blonde crossbred, body weight was also higher than Brahman Cross but lower than pure Galician Blonde, with heterosis values of 12%, 10%, 17%, and 10% at the same growth stages (Fig. 5).

Discussion

This study demonstrated a substantial level of genetic diversity among crossbred cattle populations involving Belgian Blue crossbred, Galician Blonde crossbred, and Brahman Cross breeds. The identification of five unique leptin gene sequence types in each breed suggests a high degree of genetic differentiation, providing opportunities to combine advantageous alleles related to growth and reproduction. Such diversity is important for improving traits like feed efficiency and reproductive success (Bernini *et al.*, 2023). Compared to previous studies reporting only two sequence types in Ongole cattle (Hilmia *et al.*, 2018) or seven in Bali cattle (Kurlyana *et al.*, 2023), the higher diversity observed here reflects the benefits of crossbreeding in broadening the genetic base. The application of restriction enzymes such as HindIII, BmgBI, and HpyCH4IV proved effective for SNP genotyping, with HindIII restriction enzyme in particular showing clear patterns that differentiated genotypes TT, CT,

Table 2. Frequency of restriction enzyme site which recognize the single nucleotide polymorphism locus.

Breed	Sample	Restriction enzyme										
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Brahman Cross	Type I (PQ619121)	1	5	2	8	8	17	16	2	2	1	6
	Type II (PQ619122)	1	5	2	8	8	17	16	2	2	1	6
	Type III (PQ619123)	1	5	1	7	7	17	16	1	1	0/1	6
	Type IV (PQ619124)	1	5	1	7	7	17	16	2	2	0/1	6
	Type V (PQ619125)	1	5	1	7	7	17	16	2	2	0/11	6
GBC	Type I (PQ619126)	1	5	2	8	8	17	16	2	2	1	6
	Type II (PQ619127)	1	5	2	8	8	17	16	2	2	0/1	6
	Type III (PQ619128)	1	5	1/2	8	8	17	16	1	1	0/1	6
	Type IV (PQ619129)	1	5	2	8	8	17	16	2	2	2	6
	Type V (PQ619130)	1	5	1/2	8	8	17	16	1	1	0/1	6
BBC	Type I (PQ271568)	0/1	4/5	1	7	7	17	15	2	2	1	6
	Type II (PQ271569)	0/1	4/5	1	7	7	16	16	1	1	0/1	5
	Type III (PQ271570)	1	5	1	7	7	17	16	2	2	1	6
	Type IV (PQ271571)	1	5	1	7	7	16	16	2	2	0/1	5
	Type V (PQ271572)	0/1	4/5	1	7	7	16	15	1	1	0/1	5

I=BmgBI (CAC'GTC); II=HpyCH4IV (A'CG_T);III=Xma1 (C'CCGG_G); IV=HpaII (C'CG_G); V=MspI (C'CG_G);VI=AciI (C'CG_C); VII=HpyCH4V (TG'CA);VIII=BsmBI (CGTCTCn'nnnn)_IX=Esp3I (CGTCTCn'nnnn_); X=HindIII (A'AGCT_T); XI=FauI (CCCGCnnnn'nn).

Table 3. Association of SNP g.3118 T/C with body weight (BW), body length (BL), chest width (CW), and withers height (WH) at birth (B), weaning (W), yearling (Y), and adult (A) in crossbred cattle.

Breed	Variable	Age (month)	Genotype			*P-Value*
			TT	TC	CC	
BX	BBW (kg)	Birth	26.50±1.29	27.00±1.00	27.00±1.00	0.8
	BBL (cm)	Birth	61.50±1.29	63.66±1.52	61.00±1.00	0.58
	BCW (cm)	Birth	59.50±1.29	60.00±1.00	59.00±1.00	0.79
	BWH (cm)	Birth	64.50±1.29	62.00±1.00	62.66±1.52	0.08
	WBW (kg)	6	121.75±4.35	119.66±3.51	118.66±3.21	0.57
	WBL (cm)	6	79.00±2.94	79.33±3.78	79.30±2.51	0.88
	WCW (cm)	6	72.50±1.29	72.66±2.08	74.00±1.00	0.42
	WWH (cm)	6	71.50±1.29	71.00±1.00	71.33±1.52	0.96
	YBW (kg)	12	190.75±1.70	192.00±2.00	190.33±2.08	0.56
	YBL (cm)	12	102.75±2.12	102.00±1.00	103.66±1.52	0.53
	YCW (cm)	12	106.25±1.70	104.66±2.51	104.33±1.52	0.41
	YWH (cm)	12	134.50±1.29	134.00±1.00	134.00±1.00	0.79
	ABW (kg)	18	294.00±5.35	289.00±1.00	289.00±7.00	0.37
	ABL (cm)	18	122.50±1.29	122.67±2.08	121.33±1.52	0.56
	ACW (cm)	18	119.50±1.29	119.00±1.00	120.00±1.00	0.71
	AWH (cm)	18	147.75±2.12	146.00±2.00	147.10±2.02	0.49
GBC	BBW (kg)	Birth	25.57±1.27	26.14±2.11	25.66±1.86	0.81
	BBL (cm)	Birth	58.14±2.26	60.14±3.71	60.33±3.72	0.41
	BCW (cm)	Birth	69.42±2.22	69.57±3.04	69.50±4.03	0.99
	BWH (cm)	Birth	69.00±2.70	70.28±2.42	71.50±2.42	0.23
	WBW (kg)	6	133.28±7.40^{a}	137.85±8.19^{ab}	148.00±9.77^{b}	0.01
	WBL (cm)	6	95.28±7.40^{a}	99.85±8.19^{ab}	108.33±7.76^{b}	0.02
	WCW (cm)	6	97.71±5.31^{a}	102.85±8.29^{ab}	108.33±5.46^{b}	0.03
	WWH (cm)	6	129.85±5.49^{a}	134.57±7.87^{ab}	140.00±5.17^{b}	0.03
	YBW (kg)	12	204.14±32.35	221.42±68.41	253.83±38.98	0.22
	YBL (cm)	12	118.57±6.10	112.28±9.84	116.00±8.96	0.39
	YCW (cm)	12	121.42±5.50	117.28±7.11	113.50±7.63	0.13
	YWH (cm)	12	149.42±13.50	140.57±13.20	139.00±15.83	0.36
	ABW (kg)	18	367.57±5.12^{a}	375.00±7.43^{ab}	379.16±8.25^{b}	0.02
	ABL (cm)	18	118.42±5.06^{a}	126.57±9.86^{ab}	130.83±5.77^{b}	0.02
	ACW (cm)	18	124.57±5.12^{a}	131.28±6.84^{ab}	136.66±7.81^{b}	0.01
	AWH (cm)	18	162.57±17.95^{a}	172.57±14.45^{ab}	189.66±12.04^{b}	0.01
BBC	BBW (kg)	Birth	34.61±3.81^{ab}	35.48±4.46^{b}	32.69±3.14^{a}	0.01
	BBL (cm)	Birth	71.61±3.81^{ab}	72.06±4.61^{b}	69.69±3.14^{a}	0.04
	BCW (cm)	Birth	68.61±3.81^{ab}	69.06±4.61^{b}	66.69±3.14^{a}	0.04
	BWH (cm)	Birth	74.61±3.81^{ab}	75.06±4.61^{b}	72.69±3.14^{a}	0.04
	WBW (kg)	6	132.76±14.18^{a}	172.03±17.16^{b}	150.12±18.24^{ab}	0
	WBL (cm)	6	93.71±6.79^{a}	105.51±10.90^{b}	101.01±7.65^{ab}	0
	WCW (cm)	6	84.00±14.13^{a}	122.96±17.09^{b}	100.78±18.17	0
	WWH (cm)	6	80.71±13.53^{a}	119.64±17.20^{b}	96.63±17.91^{ab}	0
	YBW (kg)	12	229.85±58.66^{a}	266.93±59.47^{b}	260.48±45.70^{ab}	0.03
	YBL (cm)	12	113.19±10.80^{a}	138.83±10.96^{b}	133.27±9.18^{ab}	0
	YCW (cm)	12	115.80±17.79^{ab}	125.38±16.64^{b}	113.36±20.53^{a}	0.03
	YWH (cm)	12	151.80±17.79^{ab}	161.38±16.64^{b}	149.36±20.53^{a}	0.03
	ABW (kg)	18	367.90±65,73	373.04±77.38	380.03±72.96	0.87
	ABL (cm)	18	134.36±7,04	135.54±7.79	137.27±8.97	0.54
	ACW (cm)	18	135.00±7,15	134.79±6.41	136.54±6.91	0.59
	AWH (cm)	18	175.00±7,15	174.79±6.41	176.45±7.42	0.64

BX (Brahman Cross), GBC (Galician Blonde crossbred), BBC (Belgian Blue crossbred). ^{a,b} Different superscript letters in the same raw indicate significantly different values at P<0.05.

and CC in Brahman Cross and other crossbred cattle. This supports its suitability as a molecular marker, consistent with the criteria proposed by Hartatik *et al.* (2015), which emphasize limited cutting sites, fragment sizes above 100 bp, and easily distinguishable band patterns. These enzymes aid in accelerating genetic improvement in cow populations by fortifying marker-assisted selection procedures (Ermawati *et al*., 2024). Additionally, the identification of leptin gene SNPs such as g.3118 T/C provides a helpful basis for the application of marker-assisted selection (MAS) in cow breeding programs. These SNPs, specifically the TC genotype in Belgian Blue crossbred (associated with superior body weight, body length, and chest girth at birth, weaning, and one year) and the CC genotype in Galician Blonde crossbred (associated with better performance at weaning and 1.5 years), can be used as molecular markers for selecting animals with desired production traits. By incorporating these SNP-based markers into breeding plans to target traits like higher carcass output and faster growth rates, farmers may accelerate genetic progress. This method is extremely significant to Indonesian beef production since crossbred cattle growth optimization can satisfy expanding market demand while preserving local condition adaption (Sutarno and Setyawan, 2016; Panjono *et al*., 2022). Applying MAS based on leptin gene SNPs in cow farming across various management systems can improve sustainability, lower breeding costs, and expedite the selection process. Furthermore, as greater genetic variety in crossbred cattle fosters stronger hybrid vigor, the adoption of MAS can optimize the genetic benefits of crossbreeding by combining with heterosis effects. Belgian Blue crossbred cattle were consistently heavier at birth, weaning, one year, and 1.5 years than Brahman Cross cattle. With heterosis values ranging from 27% to 33%, heterosis analysis revealed considerable paternal genetic effects, while pure Belgian Blue performance was still superior. Galician Blonde crossbred cattle showed positive but lower heterosis values, ranging from 10% to 17% across growth stages, which is consistent with previous research that shows crossbreeding improves offspring performance in comparison to one parental breed but does not always surpass the other (Coopman *et al*., 2007). Heterosis leveraging crossbreeding methods and SNP based MAS are part of an integrated plan to boost Indonesian beef cattle output.

In Belgian Blue crossbred carrying the TC gene showed better growth characteristics at birth, weaning, and one year of age. In contrast, Galician Blonde crossbred cattle with the CC genotype showed better performance. This combination produces a heterosis effect that allows F1 cattle to grow faster and have larger bodies from birth to one year of age. However, around the age of 1.5, the benefit of the TC genotype is no longer significant. This phenomenon might be hereditary in nature because the heterosis effect from Belgian Blue is more pronounced in the early growth phase but decreases with maturity. Data demonstrate strong heterosis values at an early age (27-33%) but collapse after 1.5 years, suggesting that the contribution of superior genes from the parents decreases as the influence of environmental factors increases (Forutan *et al*., 2024). These findings highlight the advantages of crossbreeding to boost genetic resources and capitalize on heterosis effects, particularly through the paternal line. The results show that the impact of heterosis on growth performance can be maximized by selecting superior paternal breeds, such as Belgian Blue. Similar findings about the importance of the leptin gene's TC genotype in relation to body weight, body length, and chest circumference have also been reported for Madura cattle (Kuswati *et al*., 2022). Additionally, Ongole cattle with the TC genotype of the leptin gene had a higher birth weight and a larger chest circumference (Fathoni *et al*., 2019). Additionally, the average daily increase and birth weight of Nellore cattle with the TC genotype were higher (Silva *et al*., 2014). In Simmental cattle, genotypes containing the C allele (CC or TC) of the E2-169 T>C (C57R) SNP were linked to higher body weight (Tian *et al*., 2013). Furthermore, genotypes with the C allele (CC or TC) exhibited substantial impacts, and the R25C SNP was associated with increased carcass weight in Wagyu cattle (Kawaguchi *et al*., 2017). The single nucleotide polymorphism g.12238G>A in the leptin gene was significantly associated with body length in Rambon cattle (P<0.05) (Prihandini *et al*., 2025). Similar strong correlations were found between the Lep03.4 SNP and the average daily rise in body weight at 16 months of age, indicating that genetic variation at this locus may be essential in regulating performance qualities in Colombian cattle (Martinez *et al*., 2016). Overall, this study supports crossbreeding combined with marker-assisted selection as a viable approach to boost Indonesia's beef cattle production. Further research is needed to validate the functional roles of the identified SNPs and assess their long-term effects on cattle production and adaptation under different management systems.

Conclusion

These findings suggest that crossbred cattle, particularly Belgian Blue crossbred and Galician Blonde crossbred, exhibit higher levels of genetic variation and heterosis effects when compared to Brahman Cross cattle. The restriction enzyme HindIII is a useful molecular marker for detecting genetic variation in the leptin gene. The TC genotype in Belgian Blue crossbred cattle was associated with superior growth traits at birth, weaning, and one year of age, while the CC genotype in Galician Blonde crossbred cattle showed better performance at weaning and 1.5 years, further illustrating the influence of both genotype and heterosis on growth traits in crossbred cattle.

Acknowledgments

The study materials were provided by PT Pasir Tengah, located in Cianjur, West Java, Indonesia. This research was funded by the PMDSU 2023 program through contract numbers 18/E5/PG.02.00.PL/2023 and 2203/UN1/DITLIT/Dit-Lit/PT.01.03/2023, The research was partialy funded by Rekognisi Tugas Akhir (RTA) from Universitas Gadjah Mada with Assignment Letter Number 5286/UN1.P1/PT.01.03/2024. Special thanks are extended to Retno Setyawati for her invaluable assistance in the laboratory analysis.

Conflict of interest

The authors have no conflict of interest to declare.

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