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Polymorphism of Bovine Growth Hormone Receptor Gene (g.3338A>G) and Its Association with Body Measurements and Body Weight in Pasundan Cows

W.P.B. Putra*, P.P. Agung, S. Anwar, & S. Said
Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI)
Jalan Raya Bogor-Jakarta Km. 46, Cibinong, Bogor, West Java 16911, Indonesia
*Corresponding author: widya.putra.lipi@gmail.com
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ABSTRACT

Bovine growth hormone receptor (bGHR) gene was one of the growth hormone family genes that important for body growth and development of cattle. This research was carried out to identify the polymorphism in the exon 10 of bGHR gene from 142 heads of Pasundan cows at West Java and its association with body length (BL), withers height (WH), heart girth (HG), and body weight (BW). There are two mutation points in the bGHR gene based on the sequencing analysis i.e. g.3322del.A and g.3338A>G. The single nucleotide polymorphism (SNP) at g.3338A>G was detected using PCR-RFLP method with *Alu*I restriction enzyme and showed three genotypes of AA (0.49), AG (0.37), and GG (0.14). The allelic frequencies were 0.67 (A) and 0.33 (G). The number of allele effective (ne) value was 1.79 and described that A allele was the higher allele in the bGHR gene in this study. The polymorphic informative content (PIC) value was 0.34 and categorized as a moderate category. The Chi-square ($\chi 2$) analysis showed that the population sample was in the Hardy-Weinberg equilibrium ($\chi 2<5.99$). It was concluded that the polymorphism of bGHR gene had a significant association ($p<0.05$) with BL, WH, and BW of Pasundan cows. Research showed that all body measurements in GG genotype animals were lowest than other genotypes. Meanwhile, the highest of BW was showed in GG genotype. In addition, the average of BL, WH, HG, and BW in Pasundan cows (2 PPI and 3PPI) were 126.88±14.25 cm; 133.97±31.69 cm; 145.35±13.56 cm and 201.85±44.87 kg, respectively.

Keywords: body weight, bGHR gene, body measurements, Pasundan cows, polymorphism

INTRODUCTION

The beef meat production in Indonesia at the year 2017 was 3,344,206 ton and about 23% (780,778 ton) was produced in West Java province (KEMENTAN RI, 2017). In addition, the number of native cattle at West Java year 2013 was 50,000 heads (Dwitresnadi *et al.,* 2014) and about 13% from the total number of beef cattle in West Java in the same year (382,949 heads). The Pasundan cattle are one of the genetic resources in Indonesia that adapt well at West Java and had high genetic similarities with Madura cattle (*Bos indicus*) based on microsatellite analysis (Agung *et al.,* 2019). Most of Pasundan cattle were managed on the extensive traditional system and reared as beef cattle by the farmers at West Java. There are many various body measurements (Sulasmi *et al.,* 2017) and phenotypic characterization (Said *et al.,* 2017) in the Pasundan cattle.

Selection in cattle can be conducted based on single nucleotide polymorphism (SNP) in some candidate gene affecting of growth traits. Bovine growth hormone receptor (bGHR) gene was also widely used as the candidate gene in cattle (Waters *et al.,* 2011). The bGHR gene expressed in the liver and mediated by growth hormone (GH) gene (Zhou & Jiang 2005). The length of bGHR gene in cattle was 25.688 bp and located at chromosome 20 with nine introns and ten exons (Jiang & Lucy, 2001; Lucy *et al.,* 1998; Lin *et al.,* 2009).

Previous studies reported that polymorphism of bGHR gene was occured in *Bos javanicus* breed (Maskur *et al.,* 2014; Zulkharnaim *et al.,* 2010), *Bos taurus* breeds (Di Stasio *et al.,* 2005; Olenski *et al.,* 2010; Misrianti *et al.,* 2011; Akad *et al.,* 2012) and *Bos indicus* breeds (Zulkharnaim *et al.,* 2010; Deepika & Salar, 2013). One of the polymorphism in bGHR gene were detected in exon 10 (Hadi *et al.,* 2015; Martinez *et al.,* 2016). The single nucleotide polymorphism (SNP) in the exon 10 of bGHR gene was detected in position g.3338A>G (transition mutation) and affecting the amino acid change from Serine to Glycine (Deepika & Salar, 2013; Martinez *et al.,* 2016). The previous study reported that this SNP also occurred in some Indonesian native cattle such as Bali, Pesisir (Zulkharnaim e*t al.,* 2010) and Friesian Holstein (Misrianti *et al.,* 2011).

The body measurements and body weight of cattle are important traits affecting income to farmers or breeders. Body measurements of heart girth had high correlation value with body weight (Kashoma *et al.,* 2011; Serkan & Bozkurt, 2009; Alsiddiq *et al.,* 2010). In cattle, the high heritability (h^2) value were reported in

heart girth (Khan *et al.*, 2016) and body weight (Putra e*t al.*, 2014; Gunawan & Jakaria, 2011; Kaswati e*t al.*, 2013; Hartati *et al.*, 2015; Putra *et al.*, 2018). High h^2 value in both traits reveals that these traits can be increased through selection program. Moreover, the ages of cattle are important in the selection program. In the smallholders, the ages of cattle can be detected through incisors observation (Torell *et al.*, 2003).

There are few studies regarding explain the genetic characterization in Pasundan cattle. Moreover, the effect of genetic polymorphism to productivity traits of Pasundan cattle so far was not reported. Based on the above consideration, this study was conducted to identify the bGHR/*Alu*I gene polymorphism using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and sequencing methods, and to evaluate their influence with body measurements (body length, withers height and heart girth) and body weight in Pasundan cows of West Java.

MATERIALS AND METHODS

Animals and Research Site

A total of 142 heads of Pasundan cows from 4 regions at West Java province i.e. Ciamis, Majalengka, Tasikmalaya and Pangandaran were used for blood sampling purpose (Figure 1). The exterior characteristic of animals studied: solid reddish brown color on the coat; black color on the eyelid, horn, hoof, muzzle and switch of tail; reddish-brown color on mouth lash, legs and rump patch; small or large of dewlap size; presence or absence of backline. The identification of age in the animals was performed using pair of permanent incisors (PPI) status i.e 1 PPI (1.5-2.0 years age), 2 PPI (2.5-3.0 years age), 3 PPI (3.5-4.0 years age) and 4 PPI (>4.5 years age).

Management of Animals

Most of the cattle were kept on semi-intensive management system. Cattle were pastured in the forage every day from morning (09.00 am) to evening (16.00 pm). The concentrate was given about 2 kg/head/day before pastured and after pastured. The king grass (*Pennisetum purpureum*) and rice straw were given approximately 5 kg/head/day in the evening after pastured. Fresh water was provided *ad libitum* in the colony stall. Artificial insemination (AI) was performed in the research site with Pasundan bull straw. Regular vaccination and medication were performed by veterinarian officer.

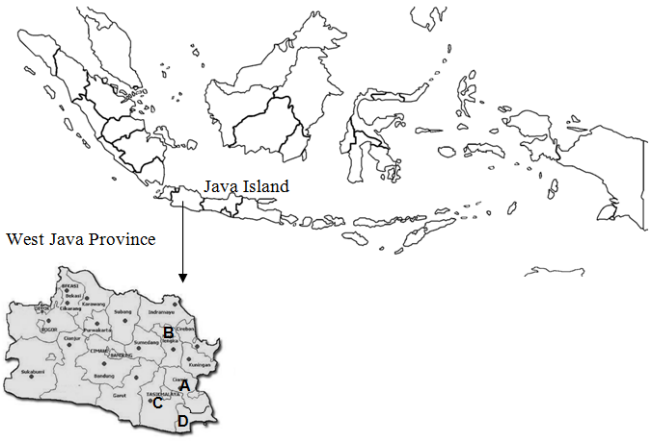

Figure 1. Location of the research site at 4 regencies at West Java Province consisted of Ciamis (A), Majalengka (B), Tasikmalaya (C), and Pangandaran (D).

Blood Samples and DNA Extraction

The blood collection procedure in animals studied was conducted according to Shabbir *et al.* (2013). The blood samples were taken from the coccygeal vein (3-5 mL) using venoject and collected in vacutainer tubes containing anticoagulant (K2EDTA). Therefore, the blood samples were used for DNA extraction analysis using the Genomic DNA Mini kit (Geneaid Biotech Ltd., Taiwan) belonging the analysis instruction. The blood samples were kept at 4-5 °C for further use.

PCR-RFLP

The polymorphism of bGHR gene was identified using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). Amount of 21 µL of PCR reagent per sample was consisted of 7.38 µL of PCR master mix (NEXpro™ Pfu, Korea), each 2.40 µL of forward and reverse primers (100 µM), 1.50 µL of DNA samples and 7.32 µL of ddH_2O. Two primers suggested by Andreas *et al.* (2010) i.e. GHR-F: 5′-CGC TTA CTT CTG CGA GGT AGA CGC-3′ and GHR-R: 5′-GTC TGT GCT CAC ATA GCC AC-3′ were used to amplify a 298 bp of fragment according to GenBank: EF207442 (Figure 2). The PCR reagent was performed using Mastercycler® (Eppendorf, Germany) with predenaturation temperature at 94°C for 5 min and following 30 cycles of denaturation at 94°C for 25 s, annealing at 63°C for 25 s, extension at 72°C for 25 s and final extension at 72°C for 5 min. Amount of 7 µL RFLP reagent per sample consisted of 0.28 µL of *Alu*I restriction enzyme (5′... AG|CT...3′), 0.70 µL of enzyme buffer 10x, 4.20 µL of PCR product and 1.82 µL ddH_2O. Therefore, the RFLP reagents were incubated at 37°C for 1 h and inactivation at 80°C for 10 min. The digested fragments were electrophoresed on 2% agarose gel (Vivantis, Malaysia) and stained with GelRed staining (Biotium, USA). Visualization of digested fragments was performed with GBOX Documentation System (Syngene, UK).

Sequencing

The DNA sequencing was performed using two PCR product of AA and GG genotypes (30 µL per sample). The sequencing analysis was performed using ABI PRISM 96-capillary 3730xl DNA Analyzer (Applied Biosystem, USA) through commercial laboratory service (First BASE Laboratories, Malaysia). The Pasundan cattle DNA sequences were aligned and compared with *Bos taurus* bGHR gene sequence (GenBank: EF207442) using MEGA ver 6.0 programs (Tamura *et al.*, 2013). The

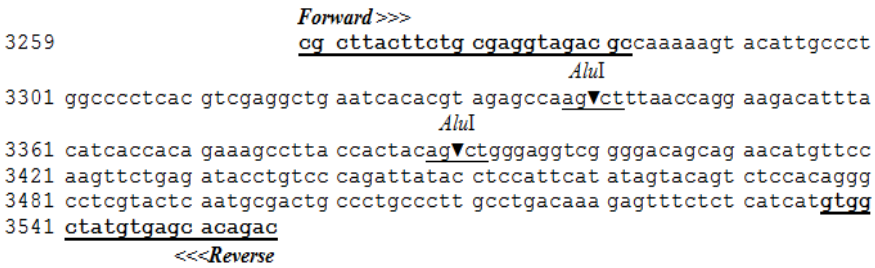

Figure 2. Primer position (underline) and *Alu*I restriction site (arrow underline) in the bGHR gene according to GenBank: EF207442.

sequencing analysis in this study was needed to confirm the mutation in the part of exon 10 in bGHR gene.

Body Measurements, Body Weight, and Ages Identification

Body measurements in the animal studied consisted of body length (BL), withers height (WH) and heart girth (HG) as presented in Figure 3. According to Ozkaya *et al.* (2016), data of BL was obtained by measuring the distance between the shoulder joint (*later tuberosity of humerus*) to the edge of the pelvic bone. Data of WH was obtained by measuring the distance from the withers to the surface by the perpendicular line. Data of HG was obtained by circling the measuring tape on scale 1 cm (Rondo, Switzerland) on the chest at the fourth rib. Data of BL and WH were measured using measuring stick on scale 1 cm. Data of body weight (BW) was obtained using a digital weighing scale. The ages identification of cattle were obtained through the number of pairs of permanent incisors (PPI) observation. According to Torell *et al.* (2003), the number of PPI in cattle were classified into 0 PPI (less than 1 years age), 1PPI (18-24 months of age), 2 PPI (30-36 months of ages), 3 PPI (42-48 months of ages) and 4 PPI (more than 48 months of age).

Statistical Analysis

Data of PPI status and body measurements were analyzed using a linear mixed model as follows:

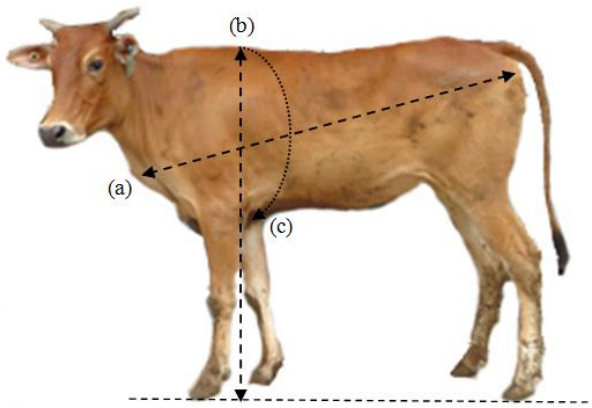

Figure 3. Scheme of body measurements for body length (a), withers height (b), and heart girth (c) of Pasundan cow.

$$Y_{ij} = \mu + P_i + G_j + e_{ij}$$

where Y_{ij} is the dependent variable (BL, WH, HG, BW); μ is the overall mean; P_i is the fixed effect of the i^{th} pairs of incisors (1 PPI, 2PPI, 3 PPI); G_j is the fixed effect of the j^{th} genotype (AA, AG, GG); and e_{ij} is the random residual effect.

The genotype and allele frequencies, observed (H_o) and expected (H_e) heterozygosity, polymorphic informative content (PIC), the number of effective allele (n_e) and Chi-square value (χ^2) were calculated to explain the genetic diversity in bGHR gene of native cows in West Java.

The value of allele frequency was calculated referring to Nei & Kumar (2000) as follow:

$$X_i = [2(N_{ii}) + (N_{ij})] / 2N$$

where X_i is the frequency of i^{th} allele; N_{ii} is the number of genotype A_iA_i; N_{ij} is the number of genotype A_iA_j; and N is the number of observation.

The observed and expected heterozygosity values were calculated referring to Weir (1996) as follow:

$$H_e = 1 - \sum_{i=1}^{n} X_i^2$$

$$H_o = \sum_{i \neq j} \frac{N_j}{N}$$

where H_e is the expected heterozygosity; H_o is the observed heterozygosity; X_i is the frequency of i^{th} allele; N_{ij} is the number of heterozygote sample; and N is the number of observation.

The polymorphic informative content was calculated referring to Hildebrand *et al.* (1992) as follows:

$$PIC = 1 - \sum_{i=1}^{n} X_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2X_i^2 X_j^2$$

where PIC is polymorphic informative content; X_i is the frequency of i^{th} allele; and X_j is the frequency of j^{th} allele.

The number of the effective allele was calculated referring to Nei & Tajima (1981) as follows:

$$n_e = \frac{1}{\sum_{i=1}^{n} X_i^2}$$

where n_e is the number of effective allele and X_i is the frequency of i^{th} allele

The Chi-square value was calculated referring to Kaps & Lamberson (2004) as follows:

$$\chi^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i}$$

Where χ^2 is Chi-square value; O_i is the number of observed i^{th} genotype; and E_i is the number of expected i^{th} genotype.

RESULTS

Polymorphism of bGHR/*Alu*I Gene

Along 298 bp of DNA fragment of bGHR was successfully amplified (Figure 4). The length of PCR product was in good agreement with the reference sequence (GenBank: EF207442). Genotyping analysis of bGHR gene was performed using *Alu*I restriction enzyme. This genotyping identified two alleles (A and G) and three genotypes (AA, AG, and GG) as presented in Figure 5. The A allele was indicated by 167, 81 and 50 bp bands, while the G allele was indicated by 167 and 131 bp bands. According to the sequencing analysis, the transversion mutation (A/G) was occurred in the exon 10 of bGHR gene of Pasundan cows at nucleotide's position of 3338 (Figure 6). According to Table 1, the PIC value was 0.34 and categorized as a moderate category. According to Nei & Kumar (2000), PIC value consisted of three categories of low (PIC<0.25), moderate (0.25<PIC<0.50), and high (PIC>0.50) (0.30<PIC<0.50). Moreover, the n_e value was 1.79 and describes that bGHR/*Alu*I in this study had two of common allele i.e. A and G. The χ^2 analysis of bGHR gene showed that the population sample was in Hardy-Weinberg equilibrium (χ^2<5.99).

Association of bGHR Gene (g.3338A>G) Polymorphism with Performance

Research showed that age had a significant effect on the body measurements of Pasundan cows in the present study (Table 2). However, association analysis showed that bGHR gene polymorphism was significantly associated (p<0.05) with the BL, WH, and BW of Pasundan cows (Table 2). The average of BL and WH in GG genotype (2 PPI and 3 PPI) were lowest compared to other genotypes and significantly difference (p<0.05). Meanwhile, the polymorphism of bGHR/*Alu*I in this study was not associated with body measurement of HG. The highest of BW value was showed in GG genotype and significant different (p<0.05).

Sequencing Results

Along 298 bp of the exon 10 region of bGHR gene were successfully to obtain based on the sequencing method (Figure 6). Two type mutations of deletion and transition were detected in the Pasundan bGHR gene at position g.3322del.A and g.3338A>G, respectively.

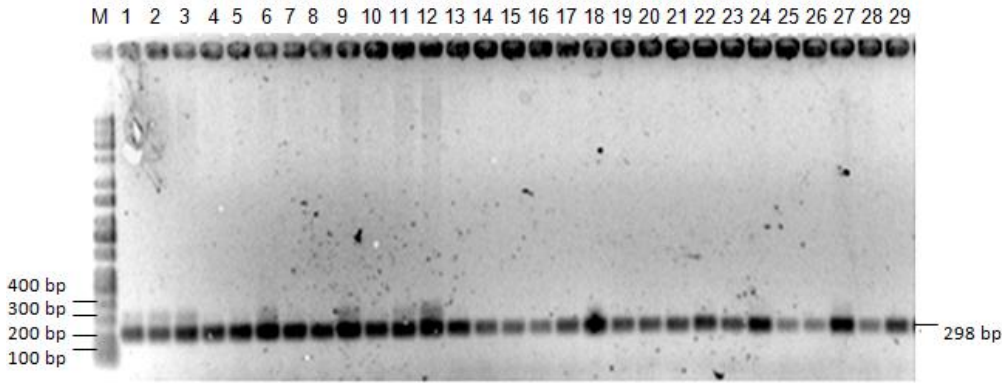

Figure 4. The amplification of bGHR gene (g.3338A>G) results on 1% agarose gel. M: marker (DNA ladder 100 bp); 1-29: number of sampel.

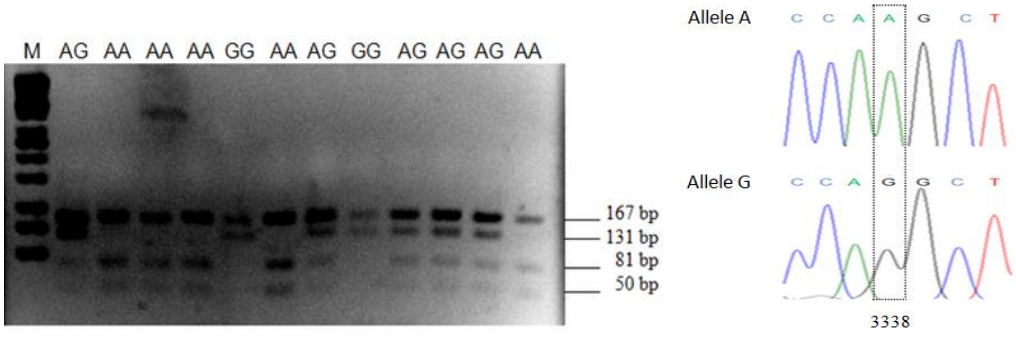

Figure 5. The fragments of bGHR/*Alu*I gene (g.3338A>G) using PCR-RFLP method on 2% agarose gel (left) showed three genotypes of AA (167 bp, 81 bp, and 50 bp); AG (167 bp, 131 bp, 81 bp, and 50 bp) and GG (167 bp and 131 bp). The sequencing result in the *Alu*I restriction site showed two type alleles of A and G (right). M: marker (DNA ladder 50 bp).

Figure 6. The sequencing results and *Alu*I restriction site (underline) in the exon 10 of bGHR gene (298 bp) in the Pasundan cattle. D: deletion at 64th base (g.3322del.A); M: mutation at 80th base (g.3338A>G); *GenBank: EF207442; M.

Table 1. The analysis results for bGHR/*Alu*I gene (g.3338A>G) polymorphism in the Pasundan cows in West Java

Genotypic frequency (N)			Allelic frequency		Heterozygosity		PIC	ne	χ2
AA	AG	GG	A	G	Expected (He)	Observed (Ho)			
0.49 (69)	0.37 (53)	0.14 (20)	0.67	0.33	0.44	0.37	0.34	1.79	3.31*

Note: N: number of observation; PIC: polymorphic informative content; Ne: number of effective allele; χ2: chi square value; *)Hardy-Weinberg equilibrium ($\chi^2_{2;\,0.05}$ = 5.99)

Table 2. Association of bGHR/*Alu*I gene (g.3338A>G) polymorphism with body measurements and body weight of Pasundan cows at 2PPI and 3PPI

Performance	Genotype (N)		
	AA	AG	GG
Body length (cm)	131.19±30.67[a] (42)	144.31±32.59[a] (26)	108.67±15.83[b] (6)
Withers height (cm)	126.16±12.89[a] (50)	129.81±17.06[a] (32)	120.44±7.33[b] (9)
Heart girth (cm)	144.50±15.20 (50)	147.38±11.53 (32)	142.89±9.97 (9)
Body weight* (kg)	188.35±22.61[a] (17)	199.17±40.49[b] (18)	227.11±28.21[c] (9)

Note: *animals from Ciamis Regency; N: number of observation; means in the same row with different superscripts differ significantly (p<0.05).

DISCUSSION

The previous study reported that A allele frequency in the exon 10 of bGHR/*Alu*I gene were higher than G allele in *Bos javanicus* (Zulkharnaim *et al.,* 2010) and *Bos indicus* breeds (Zulkharnaim *et al.,* 2010; Deepika & Salar, 2013; Martinez *et al.,* 2016). Therefore, some of *Bos taurus* breeds i.e. Friesian Holstein, Piemontese and Jersey had lower of A allele than G allele frequencies (Misrianti *et al.,* 2011; Waters *et al.,* 2011; Di Stasio *et al.,* 2005; Komisarek *et al.,* 2011). The A allele frequency of Pasundan cattle in the present study was higher than B allele and similar to the *Bos javancus* and *Bos indicus* breeds cattle.

Moderate PIC value in this study describes that the molecular selection can be performed based on SNP g.3338A/G. In addition, moderate PIC value in this study reveals that the bGHR/*Alu*I gene in Pasundan cattle are highly polymorphic. The estimation of H_o and H_e values in this study were 0.37 and 0.44, respectively and described the highly number of heterozigote genotype in the animal studied. The Hardy-Weinberg equilibrium in the population can be conducted by no selection program and random mating (Nei & Kumar, 2000).

Many studies reported that polymorphism of bGHR/*Alu*I gene was not associated with body measurements of cattle (Ardicli *et al.,* 2017a; Garrett *et al.,* 2008) and similar to the present study. Reardon *et al.* (2010) reported that genotype AG in the bGHR/*Alu*I gene had the higher of intramuscular fat percentage compare to other genotypes in Irish crossbred cattle and significantly difference (p<0.05). Hadi *et al.* (2015) reported that AG genotype in the bGHR/*Alu*I gene in the Holstein cows had the higher of calving interval, days open, milk yield and service per conception compare to AA genotype but not significantly different. Morever, Di Stasio *et al.* (2005) reported that A allele in the bGHR gene (exon 10) as the unfavourable allele on meat quality with higher

drip losses value. Fedota *et al.* (2007) reported that GG genotype in the bGHR/*Alu*I of Angus cattle had the highest of body weight at 2 and 4 years age compared to other genotypes but not significantly different. The GG genotype in the present study had the highest of BW value compare to other genotypes. In contrast, Ardicli *et al.* (2017b) reported that the polymorphism in the exon 10 of bGHR gene was not affected to the growth traits of Simmental bulls.

The frequency of GG genotype in the population studied was the lowest compared to other genotypes. Low GG genotype frequency in the Pasundan cattle can be caused by the selection system in the smallholders. In this study, the GG genotype had the highest BB value and the lowest of body measurements values. For the farmers, cattle with the lowest body measurements as the undesirable traits and will be culled. The further study to obtain the association between SNP g.3338A>G and other productivity traits in Pasundan cattle with large numbers of sample and standard management system is important to establish the marker-assisted selection (MAS).

CONCLUSION

The bGHR gene (g.3338A>G) of Pasundan cattle in this study had moderate PIC value. The GG genotype animals had the highest average body weight than other genotypes and this genotype could be used as genetic markers in Pasundan cows in the future.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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