Association of pleomorphic adenoma gene 1 with body weight and measurement of Bali cattle (Bos javanicus)

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Abstract

Background and Aim: Pleomorphic adenoma gene 1 (PLAG1) encodes a multifunctional transcription factor that controls many genes and pathways and is associated with cattle body weight and measurements. This study aimed to evaluate the association between PLAG1 polymorphisms with body weight and measurements in Bali cattle.

Materials and Methods: A total of 87 Bali cattle, consisting of 48 bulls and 39 heifers at the Breeding Center for Bali Cattle, were used as the population in this study. Cattle were 2 years old and kept semi-intensively in the pasture. Phenotype data consisting of body weight, withers height, body length, chest girth, waist height, and chest depth were measured. Birth weight data were obtained from birth records, and weight gain, adjusted weaning weight, and yearling weight were calculated using formulas. Blood samples were taken from the jugular vein as much as 5 mL, and genomic DNA was isolated using the salting-out method. Polymerase chain reaction (PCR) was performed to amplify three target polymorphisms, namely, the g.48308 C>T SNP in PLAG1, g.32212 (19 bp indel), and g.45233 T>C. The presence of a 19 bp indel was determined by direct observation of the PCR product on a 2% agarose gel. Two other polymorphisms were detected by PCR-restriction fragment length polymorphism using the restriction endonuclease enzymes SacII and BclII. PLAG1 genotype and phenotype associations were analyzed using a general linear model.

Results: The results showed that two of the target polymorphisms in PLAG1 did not vary. The DD genotype indicated by 123 bp of PCR product was the only genotype identified for g.32212 19 bp indel, and TT genotype was the only genotype found for g.45233 T>C single-nucleotide polymorphism (SNP). Conversely, the g.48308 C>T SNP was found to be polymorphic. In addition, the g.48308 C>T polymorphism of PLAG1 was significantly associated with body length of Bali cattle. Cattle with the CC genotype had a greater body length than the other two genotypes.

Conclusion: The g.48308 C>T SNP in PLAG1 was associated with Bali cattle body length characteristics. This finding could be used as a basis for selecting Bali cattle based on body length characteristics.

Keywords: association study, Bali cattle, body length, growth trait, Pleomorphic adenoma gene 1.

Introduction

Bali cattle (Bos javanicus) are indigenous Indonesian cattle, which are hypothesized to have originated from the domestication of wild Banteng long ago. Bali cattle breed well on Bali Island because the Balinese culture venerates cattle [1]. In 1986, the Government of Indonesia established the Pulukan Breeding Center which is now known as the Balai Pembibitan Ternak Unggul dan Hijauan Pakan Ternak (BPTU-HPT) Denpasar. It was founded 10 years after the Bali cattle breeding and development project started. The project focused on the improvement of Bali cattle population and its genetic quality by conventional breeding program [1]. Bali cattle have several advantageous characteristics, including good adaptability to tropical climates, high fertility, resistance to parasites, and low meat fat content [2,3]. Therefore, Bali cattle are one of the national cattle genetic resources that need to be maintained and used sustainably to optimize these advantages. Bali cattle are also listed as a cattle breed by the Food and Agriculture Organization [4].

Bali cattle are a cattle breed with the largest population in Indonesia, reaching 32.3% [5]. Therefore, Bali cattle play an important role in satisfying national meat consumption of 2.31 kg per capita, equating to the need for 624,162 tons of red meat [6]. However, this high demand is not supported by
the amount of meat produced, which only reached 515,600 tons in 2020 [7]. The low production of meat could be overcome by utilizing the potential of Bali cattle as a native Indonesian genetic resource. Improving the genetic quality of Bali cattle must be conducted continuously and in a planned manner with measurable milestones. One way to increase the productivity of Bali cattle is through marker-assisted selection (MAS). The development of molecular genetics has enabled the identification of multiple genes and genetic markers associated with genes responsible for desired phenotypic traits, including quantitative trait loci (QTL) or genomic regions affecting quantitative traits and genes for a particular trait [8,9]. A widely reported gene affecting livestock productivity is the pleomorphic adenoma gene 1 (PLAG1) gene [10].

PLAG1 is a member of the pleomorphic adenoma gene family along with PLAGL1 and PLAGL2, which express a class of zinc-finger proteins [11]. The PLAG1 encodes a multifunctional transcription factor that controls many genes and pathways, such as the Insulin-like growth factor (IGF)-II, IGF-1R, and WNT pathways [12]. Previous studies have reported that PLAG1 affects the stature and body weight of dairy and beef cattle [13,14]. Hartati et al. [10] found that a single-nucleotide polymorphism (SNP) in PLAG1 was positively associated with Indonesian Peranakan Ongole cattle body measurements. PLAG1 plays a role in controlling the increase in body measurements and height in Japanese Black cattle [15]. In addition to body measurements, polymorphisms in PLAG1 also affect cattle body weight and reproductive characteristics [16]. Xu et al. [17] reported that a 19 bp indel in PLAG1 was associated with growth traits and body measurements in Pinan, Xianan, and Jiaxian cattle in China. Zhong et al. [18] reported that the g.48308C>T polymorphism of PLAG1 significantly affected height and chest girth in five Chinese cattle breeds, and individuals with the CC genotype were preferred for these traits.

Few studies have evaluated PLAG1 in B. javanicus because of the limited characteristics of uniform cattle in a large population [19,20]. This study aimed to evaluate the association between PLAG1 polymorphisms with body weight and measurements in Bali cattle.

Materials and Methods

Ethical approval

All animal procedures related to samples of Bali cattle were approved by the Ethical Clearance Commission, National Research and Innovation Agency (BRIN) No. 81/Klirens/X/2021.

Study period and location

The study was conducted from March to November 2021. Blood samples of Bali cattle were collected at the Breeding Center of Bali Cattle (Denpasar, Indonesia). The laboratory works were carried out at the Division of Biology, Integrated Laboratory of Universitas Sebelas Maret.

Bali cattle population and measurement of phenotype data

Bali cattle used in this study were raised in the Bali cattle breeding center located at Pangyangan, Pekutatan, Jembrana Regency, Bali. The breeding center is located at an altitude of 125 m above sea level. It has an average rainfall of 485 mm/month with a temperature ranging from 22 to 30°C and average relative humidity of 70%.

A total of 87 Bali cattle, consisting of 48 bulls and 39 heifers, were obtained from two male paddocks and two female paddocks. The average age of the cattle was 2 years, and they were maintained semi-intensively with complete records. Up to 4 kg/animal of additional feed was given in the form of concentrate in the morning. Although cattle easily received forage from the paddock, additional king grass was given twice in the morning and afternoon, at a rate of 15 kg/animal. Drinking water was provided ad libitum. This management was the same for all cattle kept in the paddock. All cattle in this study had been vaccinated for Septicemia epizootica and Jembrana diseases.

Body weight data were measured using a Tru-Test EziWeigh7i digital scale (Datamars, Auckland, New Zealand) and expressed in kilograms. Body measurement data consisting of withers height (a), body length (b), chest girth (c), chest depth (d), and waist height (e) were measured using a ruler and measuring tape following SNI [21] and were calculated in centimeter (Figure-1). Birth weight data were obtained from records at BPTU-HPT Bali Cattle. Furthermore, data on weight gain, weaning weight, and yearling weight were calculated using the formulas reported by Chenette and Frahm [22] as follows:

\[ WG = \frac{(BW_i - BW)}{AA} \]

Figure-1: Body measurement of Bali cattle according to SNI 7651-4: 2017. Line (a) represents withers height; (b) body length; (c) chest girth; (d) chest depth; and (e) waist height.
Where, WG is weight gain, BWi is actual body weight, BW is birth weight, WW is adjusted 205 d weaning weight, AW is actual body weight, YW is adjusted 365 days yearling weight, AA is actual age (d), and WA is weaning age. Body weight was expressed in kilograms, and age was expressed in days.

**Blood sample collection and DNA extraction**

Blood samples were collected from the jugular vein of the cattle as much as 5 mL using an 18G vacuum needle in a 10 mL Vacutainer tube containing EDTA. The collected blood samples were stored in the cooling box at 0°C during transportation and kept in the refrigerator at 4°C until further analysis. DNA extraction from blood samples was conducted following the high salt method protocol by Montgomery and Sise [23]. The genomic DNA obtained was quantified using a NanoPhotometer (P-Class®, Implen, Munich, Germany); DNA concentration (ng/µL) and DNA purity were obtained by comparing the optical density at 260 and 280 nm. The concentration of genomic DNA in this study was set at a minimum of 20 ng/µL and purity greater than 1.8.

**Amplification and genotyping of PLAG1**

The amplification of PLAG1 targeted three polymorphisms, namely, g.48308 C>T, g.32212 (19 bp indel), and g.45233 T>C. Polymerase chain reaction (PCR) was conducted using a MiniAmp® Thermal cycler machine (Thermo Fisher Scientific, Singapore). The PCR reaction consisted of 12.5 µL GoTaq(R) Green Master Mix (Promega, Madison, USA), 9.5 µL nuclease-free water (1st BASE, Singapore), 1 µL of each primer (Table-1) [17,18], and 1 µL of template DNA. All materials were mixed in a PCR tube with a total volume of 25 µL. The amplification of PLAG1 was initiated by pre-denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing, and extension at 72°C for 30 s, and the reaction was completed after a final extension at 72°C for 10 min. The annealing temperature and time are presented in Table-1. The PCR products were run on a 2% agarose gel stained with ethidium bromide (Promega) using the Submarine Electrophoresis System Mufidex (Advance, Tokyo, Japan) for 30 min at 110 V. The 100 bp marker ladder (Geneaid, Taiwan) was used as the standard for the DNA band size. The agarose gel was then visualized using a Gel Documentation System (Glite UV, Pacific Image, Taiwan).

Genotyping was conducted by observing DNA bands of PCR products to detect the 19 bp indel, and digestion of PCR products was conducted using the PCR-restriction fragment length polymorphism (PCR-RFLP) technique to detect other polymorphisms. PCR-RFLP was performed according to the FastDigest SacII protocol for the g.48308 C>T SNP and Fast Digest BclI for the g.45233 T>C SNP (Thermo Fisher Scientific Inc., Vilnius, Lithuania). The individual Bali cattle genotype was determined by 2% agarose gel electrophoresis of the digested PCR product. The gel was then visualized under UV light using a Gel Documentation System. PCR products of three DNA pools were sequenced to confirm amplicon size (Apical Scientific, Malaysia).

**Statistical analysis**

Genotype and allele frequencies were calculated, and Pearson’s Chi-square test was conducted to verify the Hardy-Weinberg equilibrium (HWE) status. A general linear model was applied to evaluate the effects of PLAG1 polymorphisms on body weight and measurements using MINITAB version 14.0 software (Minitab Inc., USA). Association analysis was performed using the following model:

\[ Y_{ijk} = \mu + G_i + S_j + e_{ijk} \]

Where, \( Y_{ijk} \) is the phenotype of the \( k \)th animal, \( \mu \) is the population mean, \( G_i \) is the fixed effect of genotype, \( S_j \) is the fixed effect of sex, and \( e_{ijk} \) is the residual error associated with the \( k \)th animal. Tukey’s test was performed to determine pairwise differences among the genotypes.

**Results**

**Amplification and genotyping of PLAG1**

Three DNA fragments specific to PLAG1, 628 bp, 123 bp, and 767 bp, were successfully amplified using PCR (Figure-2). The 628 bp DNA fragment represented the g.48308 C>T SNP located in the 3’UTR region and was recognized by SacII. There were three genotypes, namely, the CC (502 bp and 126 bp), TC (628 bp, 502 bp, and 126 bp), and TT genotypes (628 bp, the fragment could not be digested by the

**Table-1:** Primer pairs of pleomorphic adenoma gene 1 used in this study.

| Polymorphism | Primer (5’ to 3’) | Annealing (°C/s) | Amplicon (bp) | RE   | Reference |
|--------------|-------------------|------------------|--------------|------|-----------|
| g. 48308 C>T | F: gcgcgtatcagtcaggacat  
R: cacctcaggggtgctctaggtttg  | 58/45 | 628 | SacII | [18] |
| g. 32212 | F: tccgaacaacaggtgagggagaaat  
R: cctttgcttgcctttccc  | 60/30 | 142/123 | - | [17] |
| 19 bp indel | F: tccgaacaacaggtgagggagaaat  
R: cctttgcttgcctttccc  | 56/45 | 767 | BclI | This study |
| g. 45233 T>C | F: gcgtgaaaggagaagacac  
R: gatcgggttataggaggggc  | 56/45 | 767 | BclI | This study |

RE = Restriction enzyme
A 19 bp indel is located in intron 3, specifically at nucleotide number 32212, of \textit{PLAG1}. This polymorphism was detected by the presence of a 19 bp indel, giving rise to three types of genotypes, namely, II, ID, and DD. Genotype II was characterized by the appearance of one band at 142 bp and the DD genotype at 123 bp, whereas the heterozygous ID produced two bands, 142 bp and 123 bp. The genotype detected in the Bali cattle population was the monomorphic DD genotype (100%). Another SNP identified in \textit{PLAG1}, namely, the g.45233 T>C SNP, located in exon 4, is a missense variant that has been successfully amplified in a 767 bp DNA fragment. However, this SNP did not vary in the Bali cattle population.

\textbf{Allele and genotype frequencies of \textit{PLAG1} in Bali cattle}

The analysis showed that the CC genotype was dominant (88.5%), with an allele frequency of 0.925 (Table-2). Furthermore, based on the HWE analysis, the Bali cattle population experienced an imbalance, as indicated by the HWE value of 15.21, which was greater than the $\chi^2$ value with $p<0.05$. Furthermore, the 19 bp indel and g.45233 T>C SNP were monomorphic. All individual Bali cattle genotypes were homozygous (DD and TT) for both polymorphisms. Thus, they could not be used for the association analyses.

\textbf{Association of \textit{PLAG1} polymorphisms with body weight and traits}

Analysis of the associations between the g.48308 C>T SNP and body size and weight in Bali cattle are shown in Table-3. Statistically, the SNP g.48308 C>T was significantly associated with body length in the Bali cattle population. Individuals with the CC genotype had a greater body length than those with the other genotypes ($p=0.004$). In addition, these SNPs tended to be significantly associated with waist height and chest depth in Bali cattle ($p=0.066$ and $p=0.067$, respectively).

\textbf{Discussion}

\textit{PLAG1} is a proto-oncogene that encodes a zinc-finger containing transcription factor and is involved in many pathways. It is located on bovine chromosome 14 (BTA14). Based on the ensemble database, \textit{PLAG1} spans nucleotides 23,330,541-23,375,751 bp on BTA14 and has five exons, four introns, and five transcripts [24]. This study is the first to determine an association between the g.48308 C>T SNP of \textit{PLAG1} and body length in a Bali cattle
Table 3: Association between the g.48308 C>T single-nucleotide polymorphism of pleomorphic adenoma gene 1 and body weight and traits in Bali cattle.

| Trait                  | CC (n=77) | CT (n=7) | TT (n=3) | p-value |
|------------------------|-----------|----------|----------|---------|
| Birth weight (kg)      | 18.94±0.28| 18.86±0.71| 19.00±1.00| 0.794   |
| Weaning weight (kg)    | 72.03±2.66| 67.66±4.30| 74.76±1.06| 0.103   |
| Yearling weight (kg)   | 113.47±4.64| 105.75±7.45| 118.27±2.27| 0.101   |
| Body weight (kg)       | 214.34±8.22| 209.00±13.80| 187.00±18.60| 0.069   |
| Weight gain (kg/day)   | 0.26±0.01 | 0.24±0.02 | 0.27±0.01 | 0.101   |
| Withers height (cm)    | 112.19±0.69| 110.14±2.37| 109.67±3.33| 0.142   |
| Body length (cm)       | 107.52±1.17| 101.86±2.65| 103.67±1.86| 0.004**|
| Chest girth (cm)       | 147.04±1.72| 147.71±3.33| 140.00±4.04| 0.109   |
| Waist height (cm)      | 111.86±0.67| 107.86±2.65| 109.50±1.50| 0.066†  |
| Chest depth (cm)       | 57.46±0.71 | 55.57±1.73 | 55.50±2.50 | 0.067†  |

SE is standard error; n is number of samples; ** indicates highly significant effect; † indicates a suggestive-significance effect.

population. Individuals with the CC genotype possess a greater body length than cattle with other genotypes. This polymorphism has previously been reported to be associated with growth traits in five breeds of cattle in China, revealing that cattle with the CC genotype had greater height and chest girth than cattle with the TT genotype [18]. However, the analysis showed that the Bali cattle population in this study was not in HWE. This can be caused by migration, mutation, recombination, or selection effects in the population [25]. The Bali cattle population used in this study was obtained from BPTU-HPT Bali cattle, which are allotted the task of providing superior Bali cattle. A selection process may have been employed by the agency considering that its task is to maintain and provide superior Bali cattle breeds for the community. In addition, Bali cattle breed well on Bali Island because Balinese culture respects cattle and this culture is not found in other parts of Indonesia [1,3,4]. This study also identified two other polymorphisms, namely, 19 bp indel and SNP g.45233 T>C, but these two polymorphisms were monomorphic and could not be associated with body weight and measurement traits. Previous research conducted by Xu et al. [17] who showed a positive association between the 19 bp indel in PLAG1 and growth traits in Pinan, Xianan, and Jixian cattle in China. Different results were reported by Peng et al. [26] who found that the 19 bp indel in PLAG1 was not associated with phenotypic traits of Xianian brown, red steppe, and Yunling cattle. PLAG1 has been widely reported to be associated with growth traits, body size or stature, and reproductive traits in various breeds of cattle [10,13-18]. It affects hip height, growth rate, carcass weight, body condition score, birth weight, and weight at different stages of age [27-30]. It is also responsible for growth physiology [31] in milk production, body size, coat color, and muscle formation in cattle [32]. The association between birth weight and PLAG1 genotype has been verified in a Friesian Holstein dairy cattle population. The results showed that PLAG1 is related to body size regulation [33]. This statement is supported by Abi Habib et al. [31] who reported that PLAG1 plays a role in growth physiology. Functional mutations in the bovine PLAG1 have also been reported to be associated with stature in beef cattle [12]. In addition, an epistatic interaction of the PLAG1 polymorphism with other genes, such as IGF2 and insulin, has been reported in cattle [34]. QTL regions that significantly affect livestock height have been mapped to a region on chromosome 14. The mapping of quantitative trait nucleotides (QTNs) to the PLAG1-CHCHD7 intergenic region shows a positive association with cattle body size [13]. Moreover, a pleiotropic QTN named bovine HD1400007259 in the PLAG1-CHCHD7 gene region of BTA14 was shown to be significantly associated with bicep and calf muscle size [35].

Selection based on genetic markers of growth traits is very effective in improving cattle performance. Bali cattle, indigenous Indonesian beef cattle, must be given more attention, and productivity must be increased through MAS [36]. Genetic marker-based livestock selection programs have been very effective because they can be conducted as early as needed; thus, they are more efficient than conventional livestock selection [37]. In addition, growth traits have a heritability of up to 0.43, which means that 43% of growth traits are affected by genetics [38]. The nature of growth is represented by body weight and measurements of livestock. This study showed that PLAG1 is one of the candidate genes responsible for growth traits of Bali cattle since g.48308 C>T SNP was significantly affected body length of Bali cattle. Therefore, the results of this study could be used as a basis for developing policies to improve the genetic quality of Bali cattle by the government through the BPTU-HPT Bali Cattle. Consequently, the function of the Bali Cattle Breeding Center as a provider of superior Bali cattle breeds could be optimized.

Conclusion

The g.48308 C>T SNP of PLAG1 was associated with the body length trait of Bali cattle. This finding could be used as a foundation for selecting Bali cattle based on body measurement characteristics using
MAS, which is much more effective and efficient than conventional phenotypic selection. However, validation in different populations with a larger number of Bali cattle should be taken into account to achieve more reliable results.

**Authors’ Contributions**

MC: Conceptualization, methodology, formal analysis, funding acquisition, supervision, and writing – review and editing. SS: Validation, investigation, data curation, writing – original draft preparation. MID: Validation, writing – original draft, and project administration. TAB: Validation and writing – original draft. YY and JR: Resources and writing – review and editing. SDV and PS: Investigation and writing – original draft. All authors read and approved the final manuscript.

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**Competing Interests**

The authors declare that they have no competing interests.

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