Diversity of SNP c.795A>G PLAG1 Gene and its Association to Birth Weight of Bali Cattle

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ABSTRAK

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Gen PLAG1 merupakan salah satu gen yang berpengaruh terhadap pertumbuhan dan ukuran tubuh. Penelitian ini bertujuan menganalisis keragaman gen PLAG1 dan asosiasinya terhadap bobot lahir pada sapi bali. Total sampel yang digunakan sebanyak 104 sampel yang terdiri dari 66 sapi bali BPTU-HPT Denpasar dan 38 sapi bali BPT-HMT Serading yang masing-masing memiliki data bobot lahir. Analisis keragaman gen PLAG1 dianalisis menggunakan metode PCR-RFLP dengan enzim restriksi *Sac1*. Frekuensi genotipe dan alel, heterozigotas, dan keseimbangan *Hardy-Weinberg* dianalisis menggunakan program *Popgen32*. Asosiasi SNP c.795A>G gen PLAG1 dengan bobot lahir pada sapi bali dianalisis menggunakan *General Linear Model* (GLM). Amplifikasi gen PLAG1 menghasilkan 776 pb produk PCR dengan 2 alel. Genotipe PLAG1 gen terdiri dari AA (562 pb dan 182 pb), AG (562 pb, 182 pb, dan 104 pb), dan GG (562 pb dan 104 pb). Berdasarkan hasil penelitian, gen PLAG1 pada sapi bali bersifat polimorfik. Frekuensi alel pada sapi bali berada pada keseimbangan *Hardy-Weinberg*. Genotipe pada SNP c795A>G gen PLAG1 berasosiasi dengan bobot lahir pada sapi bali. Alel A memiliki pengaruh terhadap bobot lahir tinggi pada sapi bali dengan bobot lahir tertinggi terdapat pada genotipe AG.

Kata Kunci: Sapi Bali, PCR-RFLP, Gen PLAG1, SNP

ABSTRACT

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PLAG1 gene is one of those that regulate growth and body size. This study aimed to look at the PLAG1 gene polymorphism and its relationship to birth weight in Bali cattle using PCR-RFLP. The total sample used was 104 samples consisting of 66 Bali cattle from BPTU-HPT Denpasar and 38 Bali cattle from BPT-HMT Serading, each of which had birth weight data. PLAG1 gene polymorphism was analyzed using PCR-RFLP and the Sac1 restriction enzyme. The genotype and allele frequencies, heterozygosity, and Hardy-Weinberg equilibrium were all examined using Popgen32. General Linear Model was used to analyze the association of SNP 795A>G PLAG1 gene with birth weight in Bali cattle. Amplification of the PLAG1 gene resulted in 776 bp fragments and two alleles. The PLAG1 gene had three genotypes: AA (562 bp and 182 bp), AG (562 bp, 182 bp, and 104 bp), and GG (562 bp, 182 bp, and 104 bp). Based on the results, the PLAG1 gene in Bali cattle was polymorphic. The alleles frequency of Bali cattle was in Hardy-Weinberg equilibrium. The SNP c.795A>G PLAG1 gene genotype were associated with birth weight in Bali cattle. The A allele is a determinant of high birth weight in Bali cattle where the AG genotype has the highest birth weight.

Key Words: Bali Cattle, PCR-RFLP, PLAG1 Gene, SNP

INTRODUCTION

Indonesia has abandoned animal genetic resources (AnGR) that have been adapted to different environmental conditions. Beef cattle that have adapted to a specific environment resulted in genetic variation within and between groups of beef cattle. One of the beef cattle in Indonesia, namely Bali cattle, has a large population outside Java, especially in the eastern islands of Indonesia (Purwantara et al. 2012). Bali cattle are example of native Indonesian beef cattle with high diversity and are a genetic resource for native Indonesian cattle (Martojo 2012). Bali cattle are native Indonesian cattle resulting from the domestication of wild bull (Garick & Ruvinsky 2015). The advantages of Bali cattle are being able to adapt to an unfavorable environment (Astiti 2018), good reproductive ability (70–90% conception rate), and a high percentage of carcass (45-57%) (Purwantara et al. 2012; Ismail et al. 2014).

The genetic resources of beef cattle can be used to fulfill the needs of Indonesian meat consumption, but this potential has not been optimally increased due to slow growth rate (Sutarno & Setyawan 2015). However, this local beef cattle, that have adapted to Indonesia's tropical environment, have the potential to be developed and improved through a selection based on their breeding values of the same traits that have high economic values and by selecting the gene that is related to the growth traits such as Pleomorphic adenoma gene 1 (PLAG1) gene (Juma et al. 2016).

PLAG1 gene is involved in encoding a transcription factor that is extensively expressed during fetal development. PLAG1 gene works by influencing essential growth-related genes such as insulin-like growth factor 1 (IGF1), insulin-like growth factor 2 (IGF2), and growth hormone receptor (GHR) (Pereira et al. 2016). IGFI is a gene that regulates animal growth and reproduction (Fortes et al. 2013) and IGF2 is one of the genes that control cattle's growth and body size of cattle (Karim et al. 2011). Several studies have found that the PLAG1 gene is associated with the growth traits of cattle such as body size (Fortes et al. 2013) and carcass weight (Song et al. 2016). In addition, a study showed that the PLAG1 gene also affects birth weight in PO cattle (Hartati et al. 2015).

Several cattle breeds have been studied for the polymorphism of the PLAG1 gene such as Chinese cattle (Zhou et al. 2019), Holstein-Friesian (HF) × Jersey cattle (Karim et al. 2011), New Zealand Holstein Friesian cattle (Littlejohn et al. 2012), and Simmental (Song et al. 2016). Furthermore, characterization of the PLAG1 gene in Bali cattle has previously been carried out using the direct sequencing method and 7 SNPs, were found in exon 2, one of which was the c.795A>G SNP (Putra et al. 2021). However, polymorphism of the PLAG1 gene in beef cattle in Indonesia has infrequently been studied using the PCR-RFLP technique, so it is necessary to study the polymorphism of the PLAG1 gene, especially at SNP c.795A>G and its association with birth weight in Bali cattle using the PCR-RFLP technique.

MATERIALS AND METHODS

Samples

This research was carried out at IPB University's Faculty of Animal Science's laboratory of animal molecular genetics. In this study 104 DNA samples were used, 66 heads of Bali cattle at BPTU-HPT Denpasar, Bali Province and 38 heads of Bali cattle at BPT-HMT Serading, NTB, Indonesia. The 104 samples of Bali cattle used were cattle that had birth weight data. The birth weight data used was obtained from secondary data owned by BPTU-HPT Denpasar and BPT-HMT Serading. In addition, Bali cattle blood samples for DNA extraction were collected by authorized veterinarians.

DNA amplification

The National Center for Biotechnology Information (NCBI) provided the primer sequence used in this study under the accession number KP966078.1. Primer3 program was used to create the primer design. In addition, Primer Stat was used to evaluate the primer design with forward and reverse primers (5'-GTT AGG CTA GCA GCT TAG C-3' and 5'-CAG ATG ATC ACC ACC CTG-3') which will amplify exon 2 (region 1) of the PLAG1 gene in the c.795A>G SNP and produced a 776 bp PCR product.

PCR Thermal Cycler from Applied Biosystems was used to perform DNA amplification. The DNA amplification condition consisted of 3 stages, denaturation, annealing, and extension according to PCR conditions that matched the PLAG1 gene fragment. First, 1 µL of the extracted DNA sample was taken and then transferred to a 0.2 mL tube. Next, a DNA amplification reagent consisting of 6.1 µL Nuclease Free Water, 7.5 µL PROMEGA Green Master Mix, 0.2 µL forward primer, and 0.2 µL reverse primer was placed in a 1.5 µL tube then homogenized. Next, PCR reagents were distributed to the DNA samples, homogenized with a rotary mixer and placed into the PCR machine. DNA amplification was carried out under conditions of 95°C predenaturation for 1 minute, 95°C denaturations for 15 seconds, 60°C annealing for 15 seconds, and 72°C extensions for 10 seconds. DNA amplification process was performed up to 35 cycles. The PCR products were then electrophoresed using 1.5% agarose gel to verify the PCR results.

Genotyping using PCR-RFLP

PLAG1 gene was genotyped using selected single nucleotide polymorphisms (SNPs) and performed using the PCR-RFLP technique. 5 µl of PCR product were transferred to a 0.2 ml tube. First, the tube was inserted, then the mixing was made. The mixture consisted of 0.7 µl SacI enzyme buffer, 0.4 µl SaqI enzyme, and 0.9 µl DW. The mixture was incubated in an incubator for 4 hours at 65°C. In addition, 2% agarose gel was used to visualize 7 µl of incubated DNA.

Data analysis

Genotype and allele frequency was observed and expected heterozygosity, and Hardy-Weinberg equilibrium were calculated using the Popgene32 program (Yeh et al. 2000). The following formula was

used to calculate genotype and allele frequency (Nei & Kumar 2000).

$$X_{ii} = \frac{\sum_{i=1}^{n} ni}{N} \qquad X_i = \frac{(2nii + \sum_{j \neq 1} nij)}{(2N)}$$

where X_{ii} is genotype ii frequency, n_i is the number of individuals of genotype ii, X_i is allele i frequency, n_{ii} is the number of individuals of genotype ii, n_{ij} is number of individuals of genotype ij, and N is the total of sample. Observed and expected heterozygosity was determined using the following formula (Weir 1996):

$$Ho = \sum \frac{n_{ii}}{N} \qquad He = 1 - \sum_{i=1}^{n} p_{1i^2}$$

where Ho is observed heterozygosity, nii is the number of heterozygous individuals, N is the number of observed individuals, He is expected heterozygosity, p_{1i} is allele, i frequency in locus I, and n is the number of allele of locus 1.

Hardy-Weinberg equilibrium was calculated using the following formula (Hartl & Clark 1997):

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

where χ^2 is the *chi-square* value, O is thenumber of observations of genotype I, and E is the number of expected genotypes i.

Association between the genotype of the PLAG1 gene and birth weight was analyzed using the General Linear Model (GLM) and calculated using Minitab19 Program with the following mathematical model (Hou et al. 2019):

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where Y_{ij} is the observation value, μ is the general mean value, G_i is the genotype effect, and e_{ij} is the error effect.

RESULTS AND DISCUSSION

PLAG1 gene amplification

Amplification of the PLAG1 gene fragment was carried out using a PCR Thermal Cycler from Applied Biosystems and produced a PCR product length of 776 bp as shown in Figure 1. The temperature in the annealing process is the optimum temperature for the primer attachment process used by the DNA cutting point during the amplification process. Temperatures ranging from 55°C to 72°C are commonly used for optimal annealing (Innis et al. 2012). In producing optimal gene amplification, PCR optimization is required to use various PCR process conditions such as the type of DNA polymerase, concentration, temperature, and time (Langga & Kuswinanti 2012).

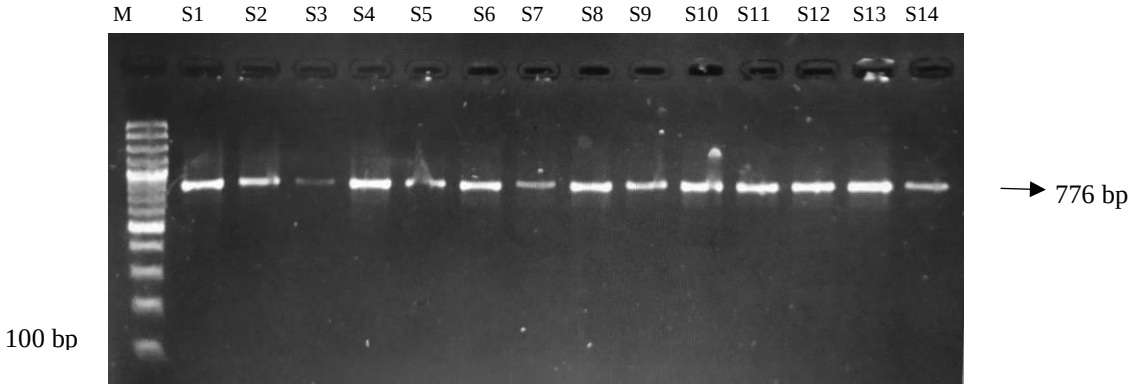

Figure 1. PCR product visualization (S1-S14: analyzed samples and M: marker 100 bp)

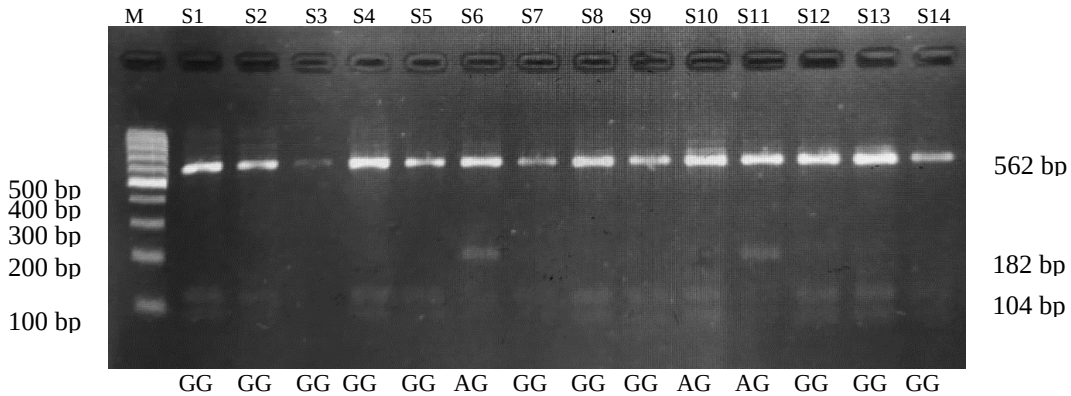

Figure 2. PLAG1 gene genotype visualization (S1-S14: analyzed samples and M: marker 100 bp)

Polymorphism of PLAG1 gene

Determination of genotype of the PLAG1 gene in Bali cattle was completed by the PCR-RFLP method using the Sac1 enzyme as a restriction enzyme. Restriction enzyme cut the DNA at certain specific sites. For example, the restriction enzyme Sac1 recognized the GAGCT|C cleavage site and cut it at 37°C. Three genotypes were produced by the results of cutting the PLAG1 gene fragment with the Sac1 enzyme. The AA genotype had two DNA fragments with product lengths of 562 bp and 182 bp. The AG genotype had three DNA fragments with product lengths of 562 bp, 182 bp, and 104 bp. Finally, the GG genotype had two DNA fragments with product lengths of 562 bp and 104 bp. DNA cleavage by restriction enzymes visualized with 2% agarose gel is presented in Figure 2.

Popgene32 program was used to calculate Bali cattle's analysis polymorphism PLAG1 gene. Table 1 shows the results of the analysis. Genotype frequency in Bali cattle at BPTU-HPT Denpasar were AA (0.03), AG (0.32), and GG (0.65), whereas at BPT-HMT Serading were AA (0.03), AG (0.16), and GG (0.82) with the highest genotype was GG in each location. In total, the genotype frequencies of AA, AG and GG in Bali cattle were 0.03, 0.26, and 0.70 with the highest genotype being GG. The G allele was the highest allele in BPTU-HPT Denpasar and BPT-HMT Serading. The highest allele frequency in Bali cattle in total was allele G (0.84). The allele of the PLAG1 gene in Bali cattle was polymorphic because its frequency was less than 0.99 (Volkandari et al. 2013). A population can be said to be polymorphic if it has more than 1 allele in 1 locus (Basyuni et al. 2012). Table 1 shows that Bali cattle observed and expected heterozygosity (Ho and He) values were less than 0.5 (50%).

Heterozygosity value that was less than 50% (0.5) indicates that a gene variation in the population is low (Dorji et al. 2012). Low gene variation in Bali cattle indicates an adverse selection or the influence of gene fixation caused by environmental factors (Rahmatullah et al. 2016). Estimating the heterozygosity value is used to calculate the genetic diversity level of a population that aims to assist the selection program. High heterozygosity indicates high genetic diversity in a population (Ulupi et al. 2014).

The Hardy-Weinberg equilibrium of the PLAG1 gene with Chi-Square (χ^2) in Table 1 shows that in Bali cattle, the PLAG1 gene frequency is in Hardy-Weinberg equilibrium because of χ^2 value < from χ^2 table (3.84). Therefore, the allele frequency in the population is relatively stable from generation to generation, the livestock population is large, and there is no mutation, selection, migration or genetic drift, gene flow, and meiotic drive, the population is in the Hardy-Weinberg equilibrium (Wang & Shete 2012). So, it is possible that the population in the two locations didn't experience migration, genetic drift, gene flow, or meiotic drive.

PLAG1 gene association with Bali cattle birth weight

Association of the PLAG1 gene with birth weight in Bali cattle was analyzed with General Linear Model (GLM) and calculated using the Minitab19 program. The results are presented in Table 2. The association analysis revealed that the SNP c.795A>G of PLAG1 gene had an association with birth weight in Bali cattle. The highest birth weight was shown in the heterozygous genotype (AG), and it had a significant effect (P<0.05) on birth weight compared to the AA and

Table 1. Genotype and allele frequencies, heterozygosity value, and χ^2 value of PLAG1 gene in Bali cattle breed

Location	N	Genotype frequency			Allele frequency		Ho	He	χ^2 value
		AA	AG	GG	A	G			
BPTU-HPT Denpasar	66	0.03	0.32	0.65	0.19	0.81	0.32	0.31	0.06^{ns}
BPT-HMT Serading	38	0.03	0.16	0.82	0.11	0.89	0.16	0.19	1.28^{ns}
Total	104	0.03	0.26	0.70	0.16	0.84	0.26	0.27	0.11^{ns}

N= Total samples, Ho= Observed heterozigosity, He= Expected heterozigosity, ^{ns}= not significant

Table 2. Association of the PLAG1 gene with birth weight in Bali cattle

Location	Genotypes			P value
	AA	AG	GG	
BPTU-HPT Denpasar	18.0 ± 1.4	20.0 ± 2.7	18.8 ± 1.8	0.08^{ns}
BPT-HMT Serading	17.0 ± 0.0^{ab}	18.2 ± 2.3^{a}	15.3 ± 1.9^{b}	0.01*

*= Significant at P<0.05, ^{ns}= Not significant. Different superscripts on the same row are different (P<0.05)

GG genotypes. The A allele is a determinant of high birth weight in Bali cattle. According to the location-based association analysis results, SNP c.795A>G of the PLAG1 gene was significantly associated with birth weight ($P<0.05$) in Bali cattle at BPT-HMT Serading. In contrast, BPTU-HPT Denpasar did not show a significant association. Another study showed that the SNP ss319607402A>G of the PLAG1 gene was associated with birth weight in New Zealand Holstein-Friesian cattle (Littlejohn et al. 2012). Furthermore, several studies stated that PLAG1 gene was associated with birth weight in PO cattle (Hartati et al. 2015) and birth weight in Brazilian Nellore cattle (Utsunomiya et al. 2013).

Birth weight is an economically essential characteristic in beef cattle, and it is usually the first trait measured in a calf (Utsunomiya et al. 2013). Genetics, feed, the cow's body weight, climate, the calf's sex, the environment experienced by the cow, and internal factors (conditions in the cow's uterus) are all factors that influence birth weight (Maylinda & Wahyuni 2020; Braz et al. 2021; Suwiti et al. 2022). The uterine environment may have an even more significant impact on fetal growth and birth weight than the parental genome (Sharma et al. 2009). However, several genes that are inherited and affect the embryo development are responses to environmental and genetic interactions experienced by the cows (Braz et al. 2021).

The difference in associations that occurred between the two locations indicated the influence of environmental conditions, namely the influence of temperature in the BPT-HMT Serading area, which was higher than the BPTU-HPT Denpasar, so that individuals with the heterozygous genotype (AG) could be better exposed and have a higher birth weight than homozygous genotypes (AA and GG). The result also indicates that the influence of genetic and environmental interactions resulted in a genotype being more adaptive in one environment than in another (Patriani et al. 2019). However, it can be seen that the birth weight at BPT-HMT Serading is smaller than at BPTU-HPT Denpasar. In order to survive the heat stress, cattle adapt to a smaller size (Syawal 2013).

PLAG1 gene is a zinc finger transcription factor of IGF2 that regulates growth and development and is found on chromosome 14 in cattle (Juma et al. 2016). PLAG1's effect on fetal growth and reproduction affecting birth weight is most likely due to the transacting regulation of the expression of insulin-like growth factors, specifically IGF2 (Voz et al. 2000). The insulin-like growth factor 2 (IGF2) gene encodes a placental and fetal growth factor that influences birth weight (St-Pierre et al. 2012).

Based on the results of this study of the PLAG1 gene's association with birth weight in Bali cattle, it is hoped that Bali cattle has the potential to be developed and increased its productivity through selection based on the breeding values of the growth traits that have high economic values, such as weaning and yearling weights. According to Boligon et al. (2009), birth weight positively correlates to growth characteristics such as weaning and yearling weights. Therefore, based on the results the AG genotype in the SNP c.795A>G of the PLAG1 gene can be used as a candidate marker assisted selection (MAS) for birth weight in Bali cattle in BPT-HMT Serading, NTB. However, further studies still need to be done to validate the results of the association to be used as a potential marker at BPT-HMT Serading with gene expression analysis.

CONCLUSION

SNP c.795A>G of the PLAG1 gene were polymorphic in Bali cattle. Furthermore, the Bali cattle's heterozygous genotype (AG) was significantly assocaited with birth weight at BPT-HMT Serading. Therefore, SNP c.795A>G of the PLAG1 gene has the potential to be used as a candidate for Marker Assisted Selection (MAS) in Bali cattle at BPT-HMT Serading.

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