

## Identification of single nucleotide polymorphism c.957A>C of PLAG1 gene and its association with growth traits in Bali cattle

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### ABSTRAK

Gen PLAG1 adalah salah satu gen yang berpengaruh terhadap sifat pertumbuhan yang terletak di kromosom 14 pada sapi. Penelitian ini bertujuan untuk mengidentifikasi SNP pada gen PLAG1 di exon 1 dan exon 2 serta asosiasinya terhadap sifat pertumbuhan pada sapi Bali. Jumlah sampel yang digunakan sebanyak 52 sampel sapi Bali, 10 sampel sapi Peranakan Ongol (PO) dan 8 sampel sapi Limousin. Identifikasi SNP gen PLAG1 dianalisis dengan metode *direct sequencing* dan *genotyping* terhadap SNP terseleksi dilakukan dengan metode PCR-RFLP. Asosiasi genotipe dari SNP c.957A>C dengan sifat pertumbuhan menggunakan pendekatan *t-test*. Ditemukan sebanyak 7 SNP di exon 2 gen PLAG1 yaitu SNP c.339A>G, c.489C>T, c.795A>G, c.957A>C, c.1023C>T, c.1056A>G, dan c.1353A>G. SNP c.957A>C divalidasi dengan teknik PCR-RFLP menggunakan enzim *TaqI* dan diperoleh tiga genotipe yaitu genotipe AA, AC, dan CC dengan frekuensi alel A dan C masing-masing 0.10 dan 0.90 pada sapi Bali, sedangkan pada sapi PO dan Limousin bersifat monomorfik. Asosiasi genotipe SNP c.957A>C gen PLAG1 tidak berpengaruh terhadap berat lahir (BW<sub>0</sub>), berat sapih (WW<sub>205</sub>), berat badan 365 hari (YW<sub>365</sub>), berat badan 730 hari (YW<sub>730</sub>), dan rata-rata pertumbuhan berat harian (ADG). SNP c.957A>C sebagai SNP spesifik sapi Bali perlu diteliti lebih lanjut sebagai kandidat penanda sifat pertumbuhan sapi Bali.

*Kata kunci: gen PLAG1, pertumbuhan, sapi Bali, SNP*

### ABSTRACT

The PLAG1 gene is one of the genes that affect the growth traits located on chromosome 14 in cattle. This study aims to obtain SNP of the PLAG1 gene in exon 1 and exon 2 and their association with growth traits in Bali cattle. The number of samples used was 52 samples of Bali cattle, 10 samples of *Peranakan Ongole* (PO), and 8 samples of Limousine cattle. Identification of SNPs PLAG1 gene was analyzed by direct sequencing method and genotyping of selected SNPs was carried out using PCR-RFLP. Association of genotypes of SNP c.957A>C with growth using *t-test*. There were 7 SNPs in exon 2 of the PLAG1 gene, namely SNP c.339A>G, c.489C>T, c.795A>G, c.957A>C, c.1023C>T, c.1056A>G, and c.1353A>G. SNP c.957A>C was validated by PCR-RFLP using *TaqI* enzyme and

obtained three genotypes, namely genotypes AA, AC, and CC with allele frequency A and C, respectively 0.10 and 0.90 in Bali cattle, while in PO and Limousine cattle were monomorphic. Genotype association of SNP c.957A>C PLAG1 gene were not associated with birth weight ( $BW_0$ ), weaning weight at 205 days of age ( $WW_{205}$ ), yearling weight at 365 days of age ( $YW_{365}$ ), yearling weight at 730 days of age ( $YW_{730}$ ), and average daily gain (ADG). SNP c.957A>C as a specific SNP for Bali cattle needs to be investigated in further research as a candidate marker for growth traits in Bali cattle.

*Keywords: Bali cattle, Growth, PLAG1 gene, SNP*

## INTRODUCTION

Bali cattle is an Indonesian cattle species which is a domesticated form of banteng (Garick and Ruvinsky, 2015). Bali cattle has several advantages, such as the ability to adapt in the unfavorable environment (Astiti, 2018), good reproduction (conception rate) approximately 70-90%), and the pretty high percentage of carcass (45-57%) (Toelihere, 2003; Purwantara *et al.*, 2012; Ismail *et al.*, 2014). However, the disadvantage of the Bali cattle is the lack of its growth (Hardjosubroto, 1994). The utilization of the Bali cattle genetic resources, both in quality and quantity is still not optimal, thus it is necessary to improve the productivity of Bali cattle. An approach that can be conducted to increase productivity and genetic quality is by the selection method (Supriyantono and Irianti, 2007; Patmawati *et al.*, 2013).

The use of DNA-based molecular genetics to help accurate selection is a perpetual effort. Exploration of potential genes, especially genes that affect growth, continues to be carried out so that they can be used as Marker Assisted Selection (MAS). So, it is hoped that in the future, the selection will be more accurate, effective and efficient (Van and Kinghorn, 2003)

Hartati *et al.* (2015) stated that the Pleomorphic Adenoma Gene 1 (PLAG1) has the highest gene expression effect on growth traits. The PLAG1 gene encodes a transcription factor that is widely expressed during fetal development that is inherited at birth. PLAG1 regulates several growth factors, including Insulin-like Growth Factor-II (IGF-II), a major regulator of body size (Karim *et al.*, 2011). Studies on the polymorphism of the PLAG1 gene have been

carried out on cattle belonging to *Bos taurus* cattle, including New Zealand Holstein-Friesian cattle by Littlejohn *et al.*, (2012), Holstein-Friesian (HF) × Jersey cattle (Karim *et al.*, 2011; Fink *et al.*, 2017) as well as Simmental cattle (Song *et al.*, 2016), and on *Bos taurus* and *Bos indicus* such as Brahman cattle and Nellore cattle (Utsunomiya *et al.*, 2017). To our knowledge, information regarding the characterization of the PLAG1 gene in Indonesian native cattle and other local cattle is still very limited. Therefore, this research needs to be carried out to identify and analyze the polymorphism of the PLAG1 gene in the exon 1 and 2 of the Bali cattle. Apart from that, this research also relates it to growth traits in Bali cattle and other cattle, such as PO cattle and Limousine cattle.

## MATERIALS AND METHODS

### Samples and Data on Growth

There were 70 blood samples used in this study, consisting of 52 samples of Bali cattle at BPTU-HPT Denpasar, 10 samples of PO cattle at *Balai Embrio Ternak* (BET) Cipelang, and 8 samples of Limousine cattle at BPTU-HPT Padang Mangatas. Blood samples were collected using a venoject needle in the bovine jugular vein. The blood sample is put into a vacuum tube containing Ethylenediaminetetraacetic Acid (EDTA). Data on growth traits of Bali cattle are obtained from BPTU-HPT in Bali Province, including data on  $BW_0$ ,  $WW_{205}$ ,  $YW_{365}$ ,  $YW_{730}$ , and ADG.

### DNA Extraction, Amplification, and Sequencing

The DNA were extracted out according to the Genomic DNA Mini Kit (Geneaid Biotech Ltd., Taiwan) procedure. Sequence data for designing primers is obtained from the National Center for Biotechnology Information (NCBI) with access number KP966078.1. The primer design was conducted using the Primer 3 program and evaluated using the Primer Stat. The primers used in exon 1 are forward primer 5'-CCA CAT GAA ACT TGA GAA GC-3' and reverse primer 5'-CCA CAT GAA ACT TGA GAA GC-3' with Polymerase Chain Reaction (PCR) products of 451 bp. The product result of PCR exon 2 (region 1) is 776 bp with forward primer 5'-GTT AGG CTA GCA GCT TAG C-3' and reverse primer 5'-CAG GGT GGT GAT CAT CTG-3'. Exon 2 (region 2) sequences used forward primer 5'-CTG AAC CTC TAC AAC ACT CC-3' and reverse primer 5'-GAA GCA GAA ACT GTG ATA CTG-3', resulting in a product length of 744 bp.

DNA amplification was performed using a PCR machine (ESCO, Singapore). The final result of the reaction has a volume of 25  $\mu$ L, each reaction uses 2  $\mu$ L of DNA, each 0.3  $\mu$ L of forward primer and reverse primer, 9.4  $\mu$ L of distillation water (DW) and 12.5  $\mu$ L of GoTaq® Green Master Mix (Promega, USA). The PCR conditions used were at predenaturation temperature of 95°C for 5 minutes, and 35 cycles for denaturation temperature at 95°C for 10 seconds, for annealing temperatures exon 1, exon 2 (region 1) and exon 2 (region 2) were 54°C, 56°C and 50°C for 20 seconds, and extension at 72°C for 30 seconds, then followed by a final extension step at 72°C for 5 minutes. A total of 3  $\mu$ L was visualized with 1.5% agarose gel with ethidium bromide. A total 22  $\mu$ L of PCR products were then sequenced using the services of 1st Base in Selangor, Malaysia, with the direct sequencing method carried out with the ABI Prism 96-capillary 3730 x 1 DNA analyzer (Applied Biosystems, USA).

### Genotyping with PCR-RFLP

The genotyping of PLAG1 gene using selected Single Nucleotide Polymorphism (SNP)

was carried out using Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) by inserting 5  $\mu$ L of PCR product into a 0.2 mL tube, then the mix was prepared. The mix consisted of 0.9  $\mu$ L DW, 0.7  $\mu$ L *TaqI* enzyme buffer, and 0.4  $\mu$ L *TaqI* enzyme. The mix was incubated at 65°C for 4 hours in an incubator. A total of 7  $\mu$ L of incubated DNA was visualized with 2% agarose gel.

### Data Analysis

The results of PLAG1 gene sequencing were identified using FinchTV 1.4.0. Dealignment sequencing samples were carried out using the Clustal W technique on MEGA X with the sequence access number Genbank KP966078.1 (Kumar *et al.*, 2018). The determination of the cutting enzyme was carried out using the NEBcutter V2.0 program (<http://nc2.neb.com/NEBcutter2/>). Genotype frequency, allele frequency, and Hardy-Weinberg's equilibrium values were calculated using the PopGen32 program (Yeh and Boyle, 1997). Data association between the polymorphism of PLAG1 genes' with selected SNPs on the growth traits of Bali cattle was analyzed using *t*-test method with the SAS 9.4 program (SAS Institute Inc, 2008).

Bali cattle live weight data has previously been corrected for age and rearing with the formula of Hardjosubroto (1994) and Khasanah *et al.* (2016) as follows:

$$ADG = \left[ \frac{BW_i - BW_0}{Age} \right]$$

$$WW_{205} = \left[ \frac{BW_i - BW_0}{Age} \times 205 \right] + BW_0$$

$$YW_{365} = \left[ \frac{BW_i - BW_0}{Age} \times 365 \right] + BW_0$$

$$YW_{730} = \left[ \frac{BW_i - BW_0}{Age} \times 730 \right] + BW_0$$

Rearing Correction =

$$\left[ \frac{\text{Average Extensive Cattle Weight}}{\text{Average Intensive Cattle Weight}} \right] \times \text{Intensive Cattle}$$

Where:

ADG = Average Daily Gain

BW<sub>i</sub> = Body weight measured from weighing-i

BW<sub>0</sub> = Birth weight

WW<sub>205</sub> = Weaning weight is corrected at 205 days of age

YW<sub>365</sub> = Yearling weight is corrected at 365 days of age

YW<sub>730</sub> = Yearling weight is corrected at 730 days of age

## RESULTS AND DISCUSSION

### Amplification and Determination SNP of PLAG1 gene

The PLAG1 gene amplification has been successfully carried out with a success rate of 100%. Amplification in the exon 1 yielded a PCR product length of 451 bp, exon 2 (region 1) produced a PCR product length of 776 bp, and the exon 2 (region 2) produced a PCR product length of 744 bp. The three fragments of the PLAG1 gene were in exon 1 and exon 2 (Figure 1) to obtain the intact fragments, and the annealing temperatures used in the fragments of exon 1, exon 2 (region 1), and exon 2 (region 2) were 54°C, 56°C, and 50°C respectively for 20 se-

conds. The amplification results can be optimized with the PCR optimization process, which is by varying the conditions used in the PCR process. The factors that influence the optimum conditions include the type of DNA polymerase, temperature, concentration (dNTPs, MgCl<sub>2</sub>, DNA polymerase, and PCR buffer), and time (Handoyo and Rudiretna, 2001).

A total of 7 SNPs were found in the PLAG1 gene in exon 2, they are SNP c.339A>G, c.489C>T, c.795A>G, c.957A>C, c.1023C>T, c.1056A>G, and c.1353A>G (Figure 2); whereas, in exon 1, SNP was not found (Figure 1). There are 6 SNPs that can be cut with restriction enzymes, they are c.489C>T (*AgeI*), c.795A>G (*SacI*), c.957A>C (*TaqI*), c.1023C>T (*NdeI*), c.1056A>G (*MseI*), and c.1353A>G (*BtsIMutI*); and 1 SNP, which is c.339A>G, there were no restriction enzymes capable of cutting the region (Table 1). Based on the 7 SNPs obtained, SNP c.489C>T is polymorphic in both types of cattle, they are Bali cattle and PO cattle; whereas SNP c.339A>G, c.795A>G, c.957A>C, c.1023C>T, and c.1056A>G are polymorphic only in Bali cattle, and SNP c.1353A>G is polymorphic at PO cattle and Limousine cattle is monomorphic at 7 SNP

Table 1. SNP, Mutation Type, and PLAG1 Gene Restriction Enzymes

Region	SNP	Mutation	Restriction Enzyme
Exon 1	-	-	-
Exon 2	c.339A>G	<i>Synonymous</i>	na
	c.489C>T	<i>Synonymous</i>	<i>AgeI</i>
	c.795A>G	<i>Synonymous</i>	<i>SacI</i>
	c.957A>C	<i>Synonymous</i>	<i>TaqI</i>
	c.1023C>T	<i>Synonymous</i>	<i>NdeI</i>
	c.1056A>G	<i>Synonymous</i>	<i>MseI</i>
	c.1353A>G.	<i>Synonymous</i>	<i>BtsI</i>

na= not analyzed

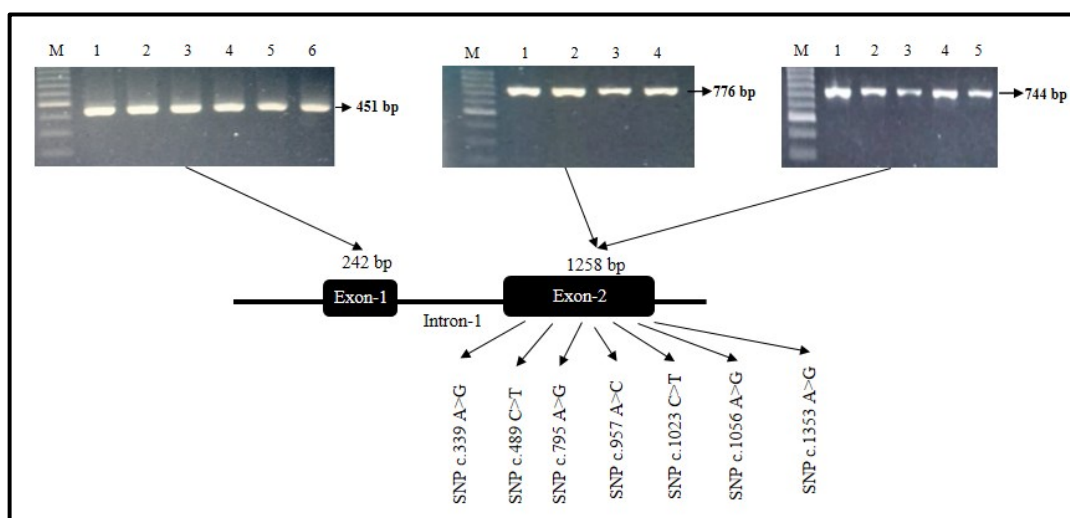


Figure 1. Visualization of PCR product at exon 1 and 2 (M = marker 100 bp and line 1-6 = analyzed samples).

points. SNPs PLAG1 gene found in Bali cattle are the specific SNPs and have never been reported in other cattle breeds. SNPs have been found in several regions of the PLAG1 gene, including SNP rs109231213 in the 3'-UTR region with the allele substitution of C to G, SNP rs210941459 (G.48308C locus) in the 3'-UTR region, and SNP ss319607402 (g.23232324A>G) in the introns region. The SNP rs109231213 was associated with body height and weight in Brahman cattle (Fortes *et al.*, 2013), and it was also associated with stature in New Zealand Dairy cattle (Karim *et al.*, 2011) and Nellore cattle (Utsunomiya *et al.*, 2017). SNP rs210941459 had a significant effect on the growth and development traits in Chinese cattle

(Zhong *et al.*, 2019). In New Zealand Holstein-Frisian dairy cattle, the SNP ss319607402 of the A allele was associated with the bodyweight of newborn calves (Littlejohn *et al.*, 2012).

#### The Allele Distribution and Hardy-Weinberg Equilibrium Analysis

The SNP c.957A>C was found in exon 2 (region 2), as the selected SNP with a high diversity compared to other SNPs in Bali cattle, as well as the cutting enzyme is simple to use for SNP c.957A>C to be associated with growth traits as a preliminary study. The SNP target can be cut with *TaqI* restriction enzyme on Bali cattle. The PCR-RFLP analysis found 3 kinds of genotypes in Bali cattle, they are AA, AC, and

Table 2. Genotype Frequency, Allele Frequency, and Hardy-Weinberg's Equilibrium, in Bali Cattle, PO Cattle, and Limousine Cattle

Cattle Order	Genotype Frequency			Allele Frequency		$\chi^2$ test
	AA	AC	CC	A	C	
Bali (n=52)	0.01	0.20	0.0.79	0.10	0.90	0.25 <sup>ns</sup>
PO (n=10)	1.00	0.00	0.00	1.00	0.00	na
Limousine (n=8)	1.00	0.00	0.00	1.00	0.00	na

n= number of animal, ns = not significant ( $P > 0.05$ ),  $X^2$ test< $X^2$ table (0.05;1) : 3.84, na= not analyzed

Table 3. Association of PLAG1 Gene with Growth Traits in Bali Cattle

Traits	Genotype			P-value	Sig.
	AA(n=1)*	AC(n=10)	CC(n=41)	AC vs CC	
BW <sub>0</sub> (kg)	17.00±0.00	19.80±2.25	19.56±2.22	0.77	ns
WW <sub>205</sub> (kg)	74.50±0.00	86.60±21.63	93.45±18.01	0.37	ns
YW <sub>365</sub> (kg)	328.8±0.00	143.97±46.35	118.85±30.63	0.13	ns
YW <sub>730</sub> (kg)	549.3±0.00	285.08±60.45	252.80±61.41	0.15	ns
ADG	0.73±0.00	0.36±0.08	0.33±0.08	0.24	ns

BW<sub>0</sub>= Birth weight, WW<sub>205</sub>= Weaning weight at 205 days of age, YW<sub>365</sub>= Yearling weight at 365 days of age, YW<sub>730</sub>= Yearling weight at 730 days of age, ADG= Average Daily Gain, n= number of animal, \*= not analyzed, Sig.=significance, ns= not significant

CC genotypes were found in 1, 10, and 41 animals respectively. Therefore, only 2 genotypes (AC and CC) were analyzed for next steps. Whereas in PO and Limousine cattles, only one kind of genotype was found, which is the AA genotype (Table 2) (Figure 3). The allele frequencies A and C in Bali cattle are 0.10 and 0.90, while the allele A frequencies in PO and Limousine cattle are 1 (Table 2). Thus, SNP c.957A>C on Bali cattle is polymorphic, while SNP on PO and Limousine cattle is monomorphic. A gene can be called monomorphic if the frequency of one of its alleles is more than 99%. This condition occurs in PO cattle and Limousines because only one allele is found, which is the A allele (Nei and Kumar, 2000; Noor, 2010; Allendorf *et al.*, 2013). The highest frequency of A allele in PO and Limousine cattle in this study was estimated due to selection and close mating management by the breeder. According to Sutikno *et al.* (2018), PO cattle were monomorphic in SNP c.-312A>G EDG1 gene which only has the A allele. It is due to the selection and mating control managed by the farmers of which the selection conducted by the breeder was to maintain cattle with A allele. Meanwhile, SNP c.957A>C on Bali cattle is in a Hardy-Weinberg equilibrium status ( $P>0.05$ ). Allendorf *et al.* (2013) state that the constant genotype and

allele frequencies from generation to generation due to gametes combining randomly in a large population cause a population to be in equilibrium. The equilibrium in the population will not change from one generation to another if there is no selection, migration, mutation, and genetic drift (Noor, 2010).

#### The Association of SNP c.957A>C with Growth Traits

SNP c.957A>C on Bali cattle is not associated with BW<sub>0</sub>, WW<sub>205</sub>, YW<sub>365</sub>, YW<sub>730</sub>, and ADG (Table 3). This might be caused by the low number of samples and the synonymous mutation. Synonymous mutations occurring in the gene-coding regions do not change the encoded proteins' amino acid composition (Sauna and Kimchi-Sarfaty, 2011). Therefore, SNP c.957A>C is classified as silence mutation. Although the AA genotype was not analyzed, the AA genotype had the highest YW<sub>365</sub>, YW<sub>730</sub>, and ADG compared to the AC and CC genotypes in Bali cattle (Table 3). In addition, all PO and Limousine cattle populations only have the AA genotype. According to Kocu *et al.* (2019) and Purpranoto (2013), the body size of PO and Limousine cattle are larger than Bali cattle. Thus, the AA genotype can be indicated as a better genotype than the AC and CC genotypes. It is hy-

pothesized that AA genotype might be a potential genotype that can be used as a candidate for genetic markers. In addition, further exploration needs to be conducted in the future regarding this matter. However, this genotype is very rare in Bali cattle (only 1 individual). Fortes *et al.* (2013) stated that the C allele of the SNP rs109231213 PLAG1 gene in Brahman cattle is positively associated with body size and adverse effect on fertility. The SNP rs136543212 in the vicinity of PLAG1 gene is a leading SNP associ-

ated with positive effect on carcass and weight traits in Nellore cattle (Pereira *et al.*, 2016). However, the SNP is different from the SNP found within this study.

The scarcity of A allele (large body weight) in Bali cattle could be caused by uncontrolled sales of large weight cattle at the plasma farmer level at BPTU-HPT Bali, where plasma farmers are small farmers who do not understand the genetic quality of livestock (Muladno, 2016). Since Bali cattle is one of the best cattle in Indo-

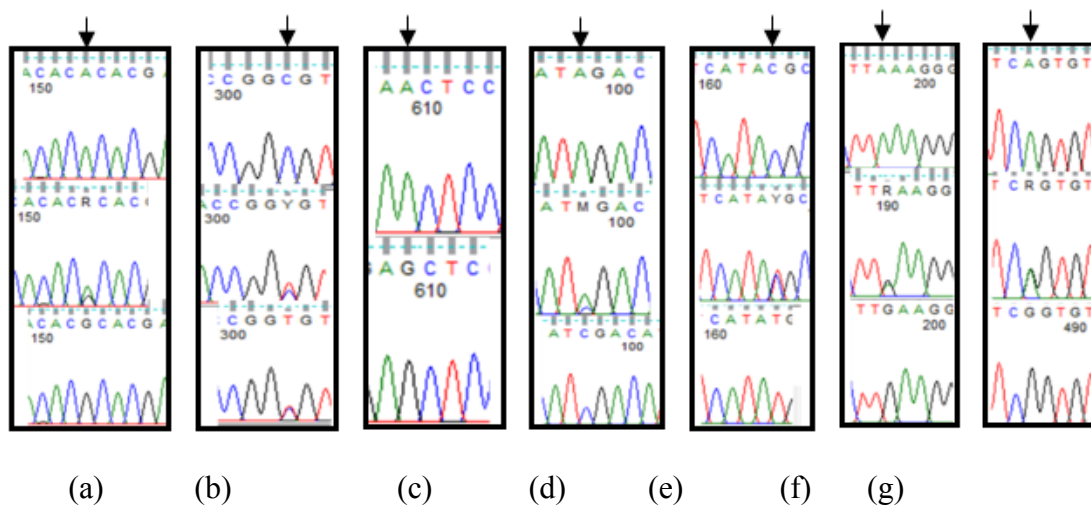


Figure 2. The determination of SNP PLAG1 gene in Bali cattle and other beef cattle. (a) c.339A>G, (b) c.489C>T, (c) c.795A>G, (d) c.957A>C, (e) c.1023C>T, (f) c.1056A>G, and (g) c.1353A>G

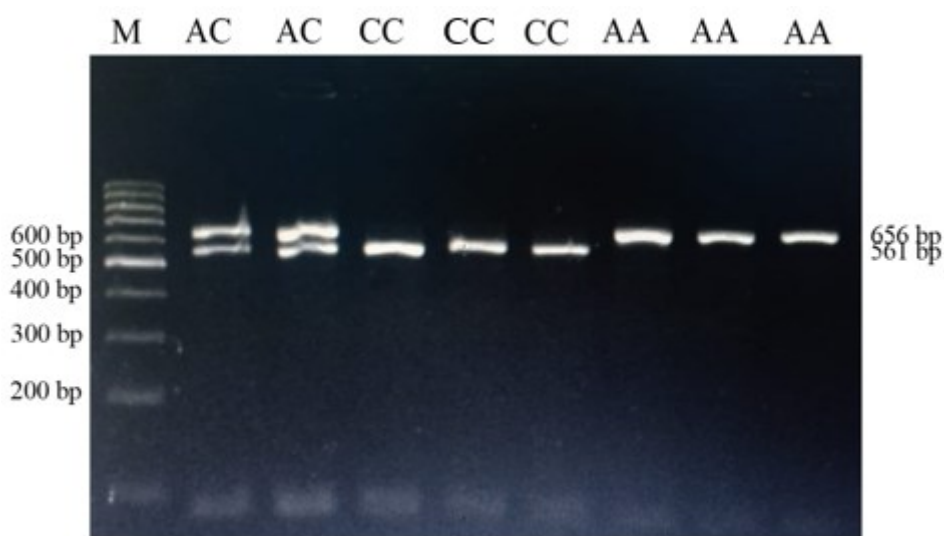


Figure 3. Visualization of PCR-RFLP SNP c.957A>C of PLAG1 gene

nesia, the demand for Bali cattle is very high.

Ardika *et al.* (2014) reported, there has been a decrease in the body size of Bali cattle as a result of negative selection. This is due to that most of the bulls are used as culling cattle and are still slaughtering productive cows. This is also supported by Romjali (2018), who stated that breeders sell good cattle to get a higher selling price in general. Thus, it is suspected that there has been a negative selection, which means that the existing cows have been mated with poor quality bulls.

The PLAG1 gene is a gene that affects growth. The PLAG1 pathway can be explained as follows. The PLAG1 gene encodes a transcription factor that is widely expressed during embryonic development. However, it is down-regulated at birth and it interacts with multiple growth factors regulating body size (Van Dyck *et al.*, 2007; Karim *et al.*, 2011). The PLAG1 gene generated a network modulating IGF1, IGF1R, IGF2, GH1, and GHR, well-known as major actors in the growth pathway (Pereira *et al.*, 2016). The SNP of PLAG1, at position g.25015640 G>T, is associated with height in Chinese cattle (Hou *et al.*, 2020). Analysis of SNP g.48308C>T PLAG1 gene showed an association with chest circumference, body length, and height in Chinese cattle (Zhong *et al.*, 2019). Karim *et al.* (2011) stated that the PLAG1 gene will transactivate the IGF-II transcription process in the promoter so that IGF-II becomes the target gene that affects growth in New Zealand Holstein-Friesian cattle. SNP genes of PLAG1 ss319607402A>G with AA genotype significantly affected the increase in body size and birth weight in New Zealand Holstein-Friesian (NZHF) cattle (Littlejohn *et al.*, 2012). Mutations that occur in the exon region will change the role of proteins, such as protein-ligand binding, protein stability, catalysis, regulation with allosterics, and other mechanisms as well as post translational protein modification (Wang and Moulton, 2001). Gunawan *et al.*, (2017) stated that mutations in the exon region will affect the polypeptide structure and protein function.

## CONCLUSION

There were 7 SNP found in exon 2 in PLAG1 gene. 6 polymorphic SNP were found in Bali cattle, while 2 SNP were found to be polymorphic in PO cattle and monomorphic in Limousine cattle; while on exon 1, no SNP was found. The SNP which have been found are SNP c.339A>G, c.489C>T, c.795A>G, c.957A>C, c.1023C>T, c.1056A>G, and c.1353A>G. SNP c.957A>C is not associated with BW<sub>0</sub>, WW<sub>205</sub>, YW<sub>365</sub>, YW<sub>730</sub>, and ADG. Therefore, SNP c.957A>C needs to be investigated further by considering the population distribution and the large number of samples to be used as a candidate for growth traits in Bali cattle.

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