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Identification of a Single Nucleotide Polymorphism at *Hinf-1* Enzyme Restriction Site of *Pit-1* Gene on Indonesian Bali Cattle Population

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ABSTRACT

This study aimed to determine the *Pit-1*|*Hinf-1* gene polymorphism in Bali cattle (*Bos javanicus*) as Indonesian native cattle and besides Madura, Pesisir, Aceh, and Katingan cattle breeds as a comparison. DNA samples were extracted from 488 blood samples consisting of Bali (245 heads), Madura (68 heads), Aceh (25 heads), Pesisir (100 heads) and Katingan (50 heads) cattle. The diversity of the *Pit-1*|*Hinf-1* gene wasere analyzed using PCR-RFLP. Whereas the nucleotide base mutations were identified by sequencing. Genotyping data were analyzed by calculating the allele frequency, observed heterozygosity (Ho) and expected heterozygosity (He) values as well as Hardry-Weinberg equilibrium test using POPGENE 1.31 program. Whereas, Tthe sequence data were analyzed by using MEGA6 program. The *Pit-1*|*Hinf-1* gene fragment analysis showed that Bali, Madura, Pesisir, Aceh, and Katingan cattle had high BB genotype, resulting in B allele frequency of 0.982, 0.963, 0.925, 0.960, and 0.960, respectively. Ho and He values were 0.074-0.130 and 0.036-0.139, respectively. Hardy-Weinberg equilibrium test did not significant for all breed populations, except for Aceh cattle population (P<0.05). Mutation from guanine (G) to adenine (A) was found in *Pit-1* gene fragment. Therefore, *Pit-1*|*Hinf-1* gene fragment had low genetic diversity in Bali cattle and other breeds population.

Key words: Bali cattle, Pit-1*|*Hinf-1 gene, polymorphism

ABSTRAK

Penelitian ini bertujuan untuk mengevaluasi keragaman gen *Pit-1*|*Hinf-1* pada sapi bali (*Bos javanicus*) sebagai sapi asli Indonesia, selain itu digunakan sapi Madura, Pesisir, Aceh, dan Katingan sebagai pembanding. Total sampel darah yang digunakan adalah dari 488 ekor, terdiri atas sapi Bali 245 ekor, sapi Madura 68 ekor, sapi Aceh 25 ekor, sapi Pesisir 100 ekor, dan sapi Katingan 50 ekor diekstraksi DNAnya. Keragaman gen *Pit-1*|*Hinf-1* dianalisis menggunakan teknik PCR-RFLP, sedangkan perubahan basa nukleotida diidentifikasi dengan teknik sekuensing. Data genotiping dianalisis dengan menghitung frekuensi gen, nilai heterosigositas dan uji kesimbangan Hardy-Weinberg menggunakan program POPGENE 1.31, sedangkan data sekuens dianalisis dengan program MEGA6. Hasil analisis fragmen gen *Pit-1*|*Hinf-1* menunjukkan bahwa sapi Bali, Madura, Pesisir, Aceh, dan Katingan memiliki genotipe BB yang tinggi, sehingga menghasilkan alel B dengan frekuensi masing-masing 0,982, 0,963, 0.925, 0,960, dan 0,960. Nilai dugaan heterosigositas observasi (Ho) dan hetersigositas harapan (He) adalah 0,074-0,130 dan 0,035-0,129. Hasil uji keseimbangan Hardy-Weinberg tidak berbeda, kecuali pada populasi sapi Aceh (P<0,05). Ditemukan perubahan basa dari guanin (G) menjadi adenin (A) pada situs pemotong enzim *Hinf-1* di gen *Pit-1*. Dengan demikian, gen *Pit-1*|*Hinf-1* memiliki keragaman genetik yang rendah pada populasi sapi Bali dan sapi lokal lainnya.

Kata kunci: sapi Bali, gen Pit-1*|*Hinf-1, polimorfisme

INTRODUCTION

Pituitary specific transcription factor-1 gene (*Pit-1*) is one of gene that has been known to be involved in controlling genes expression directly related to growth and milk production. Accordingly, it is plausible that this gene is promising to be used as a candidate of marker-assisted selection (MAS) (Bastos *et al*., 2006). Pit-1 gene has been identified and extensively studied in cattle, including *Bos taurus* (Dybus *et al*., 2003), *Bos*

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indicus/Zebu (Beauchemin *et al*., 2006; Curi *et al*., 2006; Mukesh *et al*., 2008) and *Bos primigeneus podolicus* (Salvaggi *et al*., 2011; Sevaggi & Dario, 2011). Besides, the study of this gene was also intensively conducted in native cattle breeds in some countries, including Iranian cattle (Javanmard *et al*., 2005), Southern Anatolian red cattle (Oztabak *et al*., 2008), Hanwoo cattle (Han *et al*., 2010; Seong *et al*., 2011), Turkey cattle (Ozdemir, 2012), Qinchuan cattle (Zhang *et al*., 2009) and Piemontese cattle (Ribeca *et al*., 2014). Even more, *Pit-1* gene was also identified in non-cattle species, including chicken (Nie *et al*., 2008), sheep (Bastos *et al*., 2006) and buffalo (Javanmard *et al*., 2005; Misrianti *et al*., 2010).

Cellularly, *Pit-1* gene has been known as a specific transcription factor controlling expression of growth hormone (*GH*) and prolactin (PRL) genes in pituitary anterior. Besides, *Pit-1* gene is responsively involved in protein and hormone syntheses as well as differentiation and proliferation of pituitary cells (Zhang *et al*., 2009). In genomic DNA of cattle, *Pit-1* resides in 1q21-q22 region of chromosome 1 (Woolard *et al*., 1994) which is flanked by two microsatellites DNA of TGLA57 and RM95 yielding a total of 129 amino acids consisting of 6 exons distantly spaced by 5 introns (Moody *et al*., 1995). Restriction fragment length polymorphism (RFLP) provided first evidence of high polymorphism in this gene at *Hinf-1* restriction sites, located at 5 and exon 6 (Woolard *et al*., 1994). The polymorphism, so-called *Pit-1|Hinf-1* polymorphism, is indicated by base substitution at the restriction site, in which G is changed to A (Seong *et al*., 2011). Yet, the change has no effect on the primary structure of transcripted polypeptide (silent mutation).

It is interesting that in beef cattle, *Pit-1|Hinf-1* polymorphism significantly affects growth rate of Podolica cattle (Salvagi *et al*., 2011), Hanwoo cattle (Seong *et al*., 2011) and Nanyang cattle (Kai *et al*., 2006). These evidences promoted *Pit-1|Hinf-1* polymorphism as a promising gene marker for MAS. However, some reports revealed that there was no correlation between the polymorphism and growth performance as well as their carcass traits of beef cattle (Dybus *et al*., 2003; Rogerio *et al*., 2006; Thomas *et al*., 2007; Gill *et al*., 2010). Despite abundant works, the importance of *Pit-1|Hinf-1* polymorphism was mainly deciphered based on studies on European cattle (*Bos taurus*) and zebu cattle (*Bos indicus*). To our knowledge, study on *Pit-1|Hinf-1* polymorphism on Indonesian cattle is so far limited to Bali cattle, a domesticated of Banteng (*Bos javanicus*) (Martojo, 2003) and some other Indonesian beef cattle (DGLS, 2003). Yet, comprehensive studies on the status of *Pit-1|Hinf-1* polymorphism on Indonesian cattle remain crucial factors on determining the efficiency of this gene as a marker. This work aims to observe diversity and pattern of *Pit-1|Hinf-1* polymorphism in Bali cattle, as a model of Indonesian native breed cattle, and compared with other Indonesian crossing cattle breeds through PCR-RFLP methods clarified by DNA sequencing.

MATERIALS AND METHODS

Cattle Population, Sampling and Genomic DNA Extraction

To obtain diversity and distribution patters of *Pit-1|Hinf-1* polymorphism, 488 cattle were used in this work consisting of 5 cattle breeds of Bali cattle (*Bos javanicus*) as a model of Indonesian native beef cattle and Indonesian crossed-beef cattle (Madura, Pesisir, Aceh and Katingan beef cattles) (Table 1). The cattle in this study are from different regions, including Bali island (Bali cattle), Sapudi island (Madura cattle), Sumatera island (Aceh and Pesisir cattle) and Kalimantan island (Ketingan cattle).

For genomic DNA extraction, blood samples were drawn randomly from the jugular vein by veterinarians and collected in EDTA containing-9 mL sterilized Venoject glass tubes. Genomic DNA was extracted by using Genomic DNA mini kit (blood and culture cells) (Geneaid, Taiwan) based on the protocol provided by manufacturer. Briefly, the extraction procedures consisted of five steps starting from sample preparation followed by cell lyses and DNA binding. Further steps are washing followed by DNA elution. Concentration and quality of the total genomic DNA were determined by using UV-Vis spectrophotometer. This genomic DNA was further used as a template for PCR amplification.

PCR Ampification, Genotyping, and Sequencing

The DNA fragment of Intron 5 and exon 6 from *Pit-1* gene was amplified under polymerase chain reaction (PCR) with two flanking primers as following: 5′-AAA CCA TCA TCT CCC TTC TT-3′ and 5′-AAT GTA CAA TGT GCC TTC TGA G-3′ for forward and reverse primers, respectively. The expected amplified fragment size was about 451 bp (Wollard *et al*., 1994) (Figure 1**).** For PCR, 25 μL of reaction cocktail was prepared consisting of genomic DNA as a template, 10X buffer, 10 mM dNTP, 50 mM $MgCl_2$, 30 pmol of forward and reverse primers, and 2.5 U of Taq polymerase (Promega PCR Core System USA). For the reaction, thermo cycler was set up with the following parameters: denaturation at 94 ^oC for 60 s, annealing at 60 ^oC for 45 s followed by

Table 1. The list of Indonesian beef cattle classified based on category, location (geography) and number of samples used in this experiment

Breeds	Category	Location (geography)	Number (heads)
Bali	Native	Bali Cattle Breeding Center, Bali island	245
Madura	Local	Household farmers, Sapudi island, East Java	68
Pesisir	Local	Household farmers, District of South Pesisir, West Sumatera	100
Aceh	Local	Household farmers, Aceh Provice	25
Katingan	Local	Household farmers, District of Katingan, Central Kalimantan	50

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         10         20         30         40         50         60
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
AAACCATCAT CTCCCTTCTT TCCTGCCAAC TCCCCACCTC CCAGTATTGC TGCTAAAGAC

         70         80         90        100        110        120
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
GCCCTGGAGA GACACTTTGG AGAACAGAAT AAGCCTTCCT CTCAGGAGAT CCTGCGGATG

        130        140        150        160        170        180
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
GCTGAAGAAC TAAACCTGGA GAAAGAAGTG GTGAGGGTTT GGTTTTGTAA CCGAAGGCAG

        190        200        210        220        230        240
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
AGAGAAAAAC GGGTGAAGAC AAGCCTGAAT CAGAGTTTAT TTACTATTTC TAAGGAGCAT

        250        260        270        280        290        300
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
CTCGAATGCA GATAGGCTCT CCTATTGTGT AATAGCGAGT GTTTCTACTT TTCATTCCTT

        310        320        330        340        350        360
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
TCTCTTCTCC AGCCAAAATA GAAATTAGTT ATTTGGTTAG CTTCAAAAAA TCACATCAGT

        370        380        390        400        410        420
....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
AATTTTTGCA GAAGTGTTTC TTTTCTACTT TAAAAATAAA TACAATTTAA ATTATGTTGA

        430        440        450
....|....| ....|....| ....|....| .
TGAATTATTC TCAGAAGGCA CATTGTACAT T
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Figure 1. Primary sequence of Pit-1 gene from Bos taurus (GenBank accession number: EF090615). In this sequence, restriction site of Hinf-1 is highlighted in black background (5′-G↓ANTC-3′). The underlined sequences indicated the annealing position of forward and revers primers used in this experiment.

extension step at 72 °C for 1 min. The reaction was performed for 35 cycles.

For RFLP analysis, digestion reaction using *Hinf-I* restriction enzyme (G↓ANTC) was prepared. The reaction cocktail consists of 5.0 μL endonuclease free H_2O, 2.5 μL PCR product, 2.5 μL *Hinf-I* buffer, and 0.5 μL (5 U) *Hinf-I* restriction enzyme. The reaction was performed at 37 °C for 16 h or overnight. Digestion product was observed by 2% (w/v) agarose electrophoresis in TBE with 85 V and 200 mA for voltage and current, respectively, for 45 min. For this purpose, agarose gel was prepared with 1X TBE buffer, in which 1 g of agarose was diluted in 50 mL of 1X TBE buffer. Following the electrophoresis, the band on agarose gel was visualized under UV-trans illuminator for genotyping.

Sequencing analysis of intron 5 and exon 6 fragment of *Pit-1* gene was performed on the sample showing homozygote (AA, BB) and heterozygote (AB) genotypes using both forward and reverse primers. Accordingly, 19 samples were sent to a sequencing company 1st BASE, Selangor, Malaysia, for sequencing.

Statistical Analysis

Allele frequency, observed heterozygosity (Ho) and expected heterozygosity (He) values, as well as Hardy-Weinberg equilibrium test were calculated by using POPGENE 1.31 software (Yeh *et al*., 2000). Sequencing result of intron 5 and exon 6 fragment of *Pit-1* gene was further analyzed by using MEGA6 program (Tamura *et al*., 2013).

RESULTS AND DISCUSSION

Amplifcation of *Pit-1*|*Hinf-1* gene fragment from genomic DNA of Bali, Madura, South Pesisir, Aceh and Katingan cattle, performed at 60 °C in its annealing temperature, is shown in Figure 2**.** Genotyping analysis revealed three types of genotypes, AA, AB, and BB were observed in all cattle. Specifically, BB and AB genotypes were found in Bali, Madura, Katingan and Aceh cattle, meanwhile AA genotype was found only in Pesisir cattle from West Sumatera (Figure 3). The success on gene amplification is certainly affected by annealing temperature, template DNA quality and PCR compounds (Viljoen *et al*., 2005). In this experiment, the fragment was obtained under annealing temperature of 60 °C which is slightly higher than that of reported by Woollard *et al*. (1994), which is 54 °C.

It is interesting that genotyping result revealed that AA genotype was only found in one Pesisir cattle. AA genotype was observed as a single band at about 451 bp in 2% agarose gel. Meanwhile, BB genotype was represented by two bands with 244 and 204 bp in their sizes, respectively. Three bands were observed for AB genotype for heterozygote cattle, in which the three bands (451, 244, and 207 bp) were accumulation of unmutated (wild type) and mutated bands. The fragmentation, in term of number and size, of each genotype observed in this experiment is supported by Dybus *et al*. (2013). Similar result was also reported by Zhang *et al*. (2009), Han *et al*. (2010) and Salvagi & Dario (2011) revealing that a single band was observed for AA genotype, meanwhile two- and three bands were observed for BB and AB genotypes, respectively.

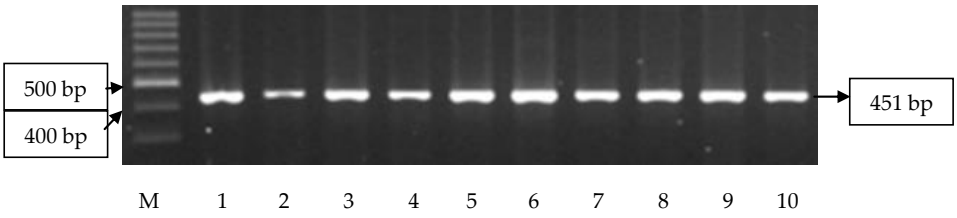

Figure 2. Amplification product of *Pit-1|Hinf-1* gene fragment observed in 2% agarose gel. The lane M corresponds to DNA ladder (marker), while lane 1-10 represent sample number in this experiment.

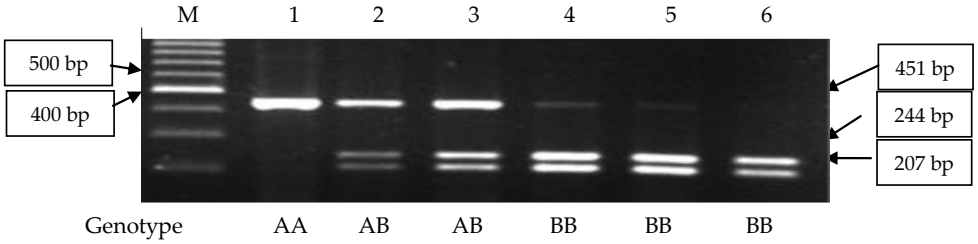

Figure 3. Digestion pattern of *Pit-1|Hinf-1* gene fragment by *Hinf-1* observed in 2% agarose gel. The lane M corresponds to DNA ladder (marker). Lane 1 is designated as AA genotype, while line 2 and 3 are AB geotype. Lane 4, 5 and 6 are classified as BB genotype.

Genotyping analysis on Bali, Madura, Pesisir, Aceh and Katingan cattle revealed that allele frequency of Pit-1|*Hinf-1* gene fragment was high for B allele, while for A ales was considerably low frequency (Table 2). Table 2 displayed the frequency for B allele was found to be higher compared to that of A allele for all cattle. In addition, there was no significant difference on the allele frequency among the cattle used in this experiment, in which the frequency for Balli, Madura, Pesisir, Aceh and Katingan were 0.982; 0.963; 0.925; 0.960; and 0.950, respectively. This result indicated that B allele is fixed in all cattle used in this experiment (Table 2). Similar result was observed in Zebu cattle (*Bos indicus*) (Beauchemin *et al*., 2006; Mukesh *et al*., 2008) (Table 3). Contradictive result was observed on European beef cattle *Bos taurus* in which the B allele frequency is decreasing as found in Angus (0.55) and Hereford (0.790) cattle (Moody *et al*., 1995) as well as Limousine cattle (0.730) (Dybus *et al*., 2003). Similar evidence of decreasing of B allele frequency was also observed in *Bos primigenius* cattle, including Podolica cattle (0.700) (Selvaggi *et al*., 2011; Selvaggi & Dario, 2011). Interestingly, as reported by Curi *et al*. (2006), B allele was found to be low in Zebu cattle (*Bos indicus*) including Nellore (0.103) and Chanchim (0.117) cattle originated from Brazil (Table 3). Besides, B allele was also found to be low in Brangus (0.181) (Thomas *et al*., 2007), Hanwoo (0.089) (Han *et al*., 2010) and cross breed of half-Angus and half-Simmental cross breed (0.000) (Curi *et al*., 2006). The existence of B allele or BB genotype has significant correlation to carcass quality specifically in term of back fat thickness, in Hanwoo cattle (Han *et al*., 2010), and intramuscular fat in Brangus bulls (Thomas *et al*., 2007). In Qinchuan cattle, AB genotype was shown to be higher than BB genotype for body

Table 2. Genotype number and allele frequency of *Pit-1|Hinf-*1 gene fragment in Indonesian beef cattle

Breeds	n	Genotype number			Allele frequency	
		AA	AB	BB	A	B
Bali	245	0	9	236	0.018	0.982
Madura	68	0	5	63	0.037	0.963
Pesisir	100	1	13	86	0.075	0.925
Aceh	25	0	2	23	0.040	0.960
Katingan	50	0	5	45	0.050	0.950

Note: n= sample number (heads).

Table 3. Allele frequency distribution of *Pit-1|Hinf-1* gene fragment in *Bos taurus, Bos indicus, Bos primigenius,* and *Bos javanicus*

Species	Breeds	n	Allele frequency		References
			A	B	
Bos taurus	Limousin	130	0.270	0.730	Dybus *et al.* (2003)
	Angus	19	0.450	0.540	Moody *et al.* (1995)
	Hereford	45	0.210	0.790	Moody *et al.* (1995)
Bos indicus	Nellore	79	0.897	0.103	Curi *et al.* (2006)
	Chanchim	30	0.883	0.117	Curi *et al.* (2006)
	Brahman	324	0.059	0.941	Beauchemin *et al.* (2006)
	Ongole	42	0.048	0.952	Mukesh *et al.* (2008)
	Hariana	42	0.114	0.886	Mukesh *et al.* (2008)
Bos primigenius	Podolica	104	0.300	0.700	Selvaggi & Dario (2011)
Bos javanicus	Bali	245	0.018	0.982	This work

Note: n= sample number (heads).

weight and shoulder height (Zhang *et al*., 2009). Yet, AA genotype was not analyzed due to limited number of samples (Zhang *et al*., 2009). In addition, there was no correlation between polymorphism in Pit-1|*Hinf-1* gene fragment and production traits in Limousine cattle (Dybus *et al*., 2003).

Observed and expected heterozygocity (Ho and He, respectively) values indicated that diversities of Indonesian native cattle (Bali cattle) and Indonesian cross-breed cattle (Madura, Pesisir, Aceh, and Katingan) were remarkably low. The values were 0.0370-0.130 and 0.036-0.139 for Ho and He, respectively (Table 4). Table 4 also showed that Ho and He values among the cattle breeds in this experiment were statistically similar. This indicated gene frequency in each population is in equilibrium state as supported by Hardy-Weinberg test in this experiment (P>0.05). Yet, Aceh cattle is an exception in which the gene frequency in this population was considerably not in equilibrium state based on the test (P<0.05). Altogether, in general, population of Indonesian cattle breeds is in dynamic equilibrium, but not for Aceh cattle population. This discrepancy might be due to limited sample number in this experiment. As Allendrof & Luikart (2007) stated, population size is one of constraint in Hardy-Weinberg equilibrium status. Other constraints are random matting, the absence of mutation, the absence of selection as well as the absence of migration. Hardy-Weinberg equilibrium status was also found in population of Zebu (Mukesh *et al*., 2008), red Anatolian (Oztabak *et al*., 2008), Qinchuan (Zhang *et al*., 2009), Hanwoo (Han *et al*., 2010) and Holstein Turkey (Ozdemer, 2012) cattle.

Sequences analysis on A and B allele diversity found in Bali, Madura, Pesisir, Aceh and Katingan cattle revealed that G base was changed to A base at the restriction site of *Hinf*-I (5-GANTC-3) located in the intron 5 exon 6 fragment of *Pit-1* gene (Figure 4). The same mutation (G→A) was also found, and classified as silent mutation, in exon 6 of *Pit-1* gene (Seong *et al*., 2011) which was speculated to have no direct effect on phenotype variation (Curi *et al*., 2006). However, the relation between *Pit-1*|*Hinf-1* polymorphism and carcass quality are significant for Hanwoo cattle (Seong *et al*., 2011; Han *et al*., 2010) as well as the relationship between the polymorphism and intramuscular fat, in Brangus bulls (Thomas *et al*., 2007) or production traits, in Qinchuan cattle (Zhang *et al*., 2009).

Table 4. Heterozygocity values and PIC of Pit-1|Hinf-1 gene fragment in Indonesian beef cattle

Breeds	n	Values		H-W test
		Ho	He	
Bali	245	0.037	0.036	(0.07) ns
Madura	68	0.074	0.071	(0.08) ns
Pesisir	100	0.130	0.139	(0.71) ns
Aceh	25	0.080	0.077	(0.02) *
Katingan	50	0.100	0.095	(0.11) ns

Note: n= sample number (heads); H-W= Hardy-Weinberg; * = significantly different (P<0.05); ns= insignificantly different (P>0.05).

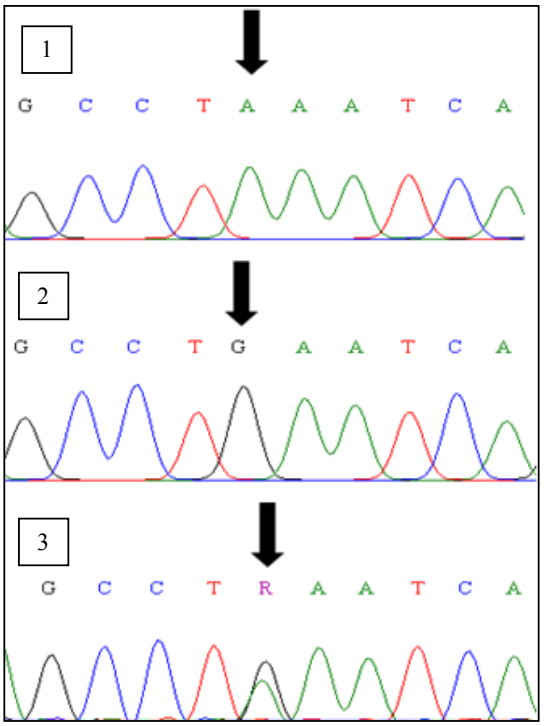

Figure 4. Sequence of *Pit-1*|*Hinf-1* gene fragment obtained from sequencing of the cattle with AA (1), BB (2) and AB (3) genotypes. The arrow indicated the base position that changes (G base to A base) due to polymorphism. R indicates either G or A base.

Altogether, this result provides evidence for the use of *Pit-1*|*Hinf-1* fragment in intron 5 and exon 6 as marker candidate for cattle growth (Zhang *et al*., 2009; Selvaggi *et al*., 2011) and carcass quality (Thomas *et al*., 2007; Han *et al*., 2010; Ribeca *et al*., 2014). Yet, the use of this allele, as well as other desired alleles in Bali cattle specifically and other local breed cattle in Indonesia remain to be formulated to obtain appropriate breeding strategy to maintain and improve the allele frequency or equilibrium in the population.

CONCLUSION

B allele of Bali cattle and other local breed cattles had been found to be low in its diversity. This allele was almost fixed in all cattles and had similar distribution pattern on *Pit-1*|*Hinf-1* gene fragment on intron 5 and exon 6. Polymorphism in this fragment was indicated by the change of G base to A base.

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