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), Pesisir (25 heads), Pesisir (100 heads) and Katingan (50 heads) cattle. The diversity of the *Pit-1|Hinf-1* gene was 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 Hardy-Weinberg equilibrium test using POPGENE 1.31 program. Whereas, The sequence data were analyzed 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 a 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

**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 javanicus* 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 javanicus*...
carrus traits of beef cattle polymorphism and growth performance as well as their ports revealed that there was no correlation between the promising gene marker for MAS. However, some re-evidences promoted also identified in non-cattle species, including chicken 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 (Ribece 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 TGLAS7 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 Hind-I restriction sites, located at 5 and exon 6 (Woolard et al., 1994). The polymorphism, so-called Pit-1|Hind-I 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|Hind-I polymorphism significantly affects growth rate of Podolica cattle (Salvaggi et al., 2011), Hanwoo cattle (Seong et al., 2011) and Nanyang cattle (Kai et al., 2006). These evidences promoted Pit-1|Hind-I 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|Hind-I polymorphism was mainly deciphered based on studies on European cattle (Bos taurus) and zebu cattle (Bos indicus).

To our knowledge, study on Pit-1|Hind-I polymorphism on Indonesian cattle is so far limited to Bali cattle, a domesticated of Banteng (Bos javanicus) (Martoto, 2003) and some other Indonesian beef cattle (DGLS, 2003). Yet, comprehensive studies on the status of Pit-1|Hind-I 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|Hind-I 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|Hind-I 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 cattle) (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 (Katingan 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 Amplification, 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 MgCl2, 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 °C for 60 s, annealing at 60 °C 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 Province | 25 |
| Katingan | Local | Household farmers, District of Katingan, Central Kalimantan | 50 |
extension step at 72 °C for 1 min. The reaction was performed for 35 cycles.

For RFLP analysis, digestion reaction using Hinf-1 restriction enzyme (Gf1ANTC) was prepared. The reaction cocktail consists of 5.0 μL endonuclease free H2O, 2.5 μL PCR product, 2.5 μL Hinf-1 buffer, and 0.5 μL 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

Amplification 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 only 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 genotype. Lane 4, 5 and 6 are classified as BB genotype.

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

| Breeds  | n  | Genotype number | Allele frequency |
|---------|----|----------------|------------------|
| Bali    | 245| AA 9 BB 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 |
|-----------|---------------|----|------------------|------------|
| 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: m= 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: 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 mating, 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 (Oztakab et al., 2008), Qinchuan (Zhang et al., 2009), Hanwoo (Han et al., 2010) and Holstein Turkey (Ozdemzer, 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-I 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  | Ho     | He     | H-W test |
|----------|----|--------|--------|----------|
| 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-I 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-I 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 cattle had been found to be low in its diversity. This allele was almost fixed in all cattle and had similar distribution pattern on Pit-1|Hinf-I 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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