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Genetic Variation of Calpastation Gene of Indigenous Bali Cattle (*Bos javanicus*) in Indonesia

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ABSTRACT

Calpastatin is one of gene markers affecting meat tenderness. The study aimed to evaluate genetic variation of calpastatin (CAST) gene of Bali cattle (*Bos javanicus*) in Indonesia. A total of 61 samples consisting of 21 Bali cattle, 22 Ongole cattle (*Bos indicus*), and 18 Friesian Holstein (FH) cattle (*Bos taurus*) were applied. The Ongole and FH cattle were involved for breed comparison. DNA was extracted from fresh blood using a High Salt method and measured their quality by a Spectrophotometer. A 523 bp of Calpastatin gene fragment was amplified by Polymerase Chain Reaction and Restriction Fragment Polymorphism (PCR-RFLP) technique with *Rsa*I restriction enzyme for genotyping. Result showed that two variants alleles (C and G) and three genotypes (CC, GC, GG) were found in those Bali, Ongole and FH samples. Allele G was dominant allele with the highest G allele was in Bali cattle population (0.88). The higher percentage of allele C was found in Ongole and Friesian Holstein compared to that in Bali cattle. The Ongole breed tends to have a potential source of lean meat quality. This finding identified that genetic variation of CAST gene was exist in Bali cattle and adapted cattle of Ongole and FH in Indonesian.

1. Introduction

Indonesia has several breeds of local beef cattle, such as Bali, Ongole (long adapted), Madura, Aceh, Pasundan cattle etc. With their carcass percentage reach 45-53% (Hafid and Rugayah 2009; Purpranoto 2013). Meat quality as described how much meat is attractive to consumers and looks good before satisfying their palate. International studies have shown that eating beef quality is assessed fairly and consistently by worldwide consumers (Polkinghorne and Thompson 2010). The aroma, juiciness, tenderness, and flavor of meat should meet to the consumer's expectation (Aberle *et al.* 2001; Thu 2006). Meat tenderness is one of important factor related to consumer acceptance and satisfaction (Jelenikova *et al.* 2008; Verbeke *et al.* 2010) that included one of the attributes of meat quality of beef cattle (Pintos and Corva 2010). Those criteria are critical issues for the meat industry at 21th century (Joo *et al.* 2013). Tenderness trait is influenced by many factors of genetics and environmental. Genomic technologies have been discovered and used as a genetic marker in livestock breeding to improve beef performance (Cafe *et al.* 2010). Quantitative Trait Loci's mapping and association studies have revealed that two genes are responsible for a high proportion of the genetic variation in beef tenderness *i.e* calpain and calpastatin (Schenkel *et al.* 2006; Pintos and Corva 2010; Ekerljung 2012).

Calpastatin enzyme is an endogenous inhibitor of calpain *i.e.*, μ- and m-calpain (Goll *et al.* 2003). High levels of Calpastatin are associated with poor quality of meat which reduce the activity of calpain due to of reducing the proteolysis process required for tender meat (Kemp *et al.* 2012). Calpastatin enzyme was encoded by the CAST gene (Nowak 2011) and located in BTA 7 (Bishop *et al.* 1997).

Several studies reported that polymorphisms of calpastatin gene was found and associated with meat quality and tenderness (Schenkel *et al.* 2006; Li *et al.*

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2010). Juszczuk-Kubiak *et al.* (2004) reported that substitution of G → C was located at position 1,460 nucleotide of exon 12. The mutation showed that Ser → Thr substitution at position 20 of amino acid sequence of CAST protein was associated with meat quality traits. Another SNP location, Schenkel *et al.* (2006) found that mutation at position 257 (G/C) of CAST gene affects the marbling score in beef cattle of Simmental, Limousin, Charolais, and Angus. At the same SNP (257 G/C), Pinto *et al.* (2010) showed that additive and dominance effect for shear force was found in all aging periods (7, 14, and 21 days of aging). Another information, Royer *et al.* (2016) reported that SNP of CAST gene (rs110496242) was a significant effect of marbling in Brahman cattle.

As one of Indonesian beef cattle, Bali cattle was recognized in 1970's due to their meat quality (Margawati *et al.* 2017). Long adapted beef cattle of Ongole breed in Indonesia was assumed to have good meat quality based on a quick glance field observation. The FH cattle was also a long adapted dairy cattle in Indonesia was used as meat source especially of FH male. The objective of this present study was to evaluate the genetic variation of Calpastatin gene in Bali cattle and compared to both adapted cattle of Ongole and FH in Indonesia.

2. Materials and Methods

2.1. Animal Samples

A total of 61 cattle samples consisting of 21 Bali cattle, 22 Ongole cattle, and 18 Friesian Holstein cattle (FH) were analysed. Blood samples (5 ml each) was taken from *vena caudalis* and collected into a vacutainer containing K_3ETA (BD Vacutainer Systems, Plymouth, UK).

2.2. DNA Extraction and Quantification

DNA from the blood samples was extracted using a High Salt method (Montgomery and Sise 1990). The concentration and purity of the DNA samples was measured by a Spectrophotometer (GeneQuant Pro, Amersham Bioscience UK). DNA samples were prepared at 50 ng/µl concentration and stored at -20°C before used.

2.3. Amplification of Calpastatin Gene

A pair primer of *Forward*/F (5'-CCT CGA CTG CGT ACC AAT TCC GAA GTA AAG CCA AAG GAA CA-3') and *Reverse*/R (5'-ATT TCT CTG ATG GTG GCT GCT CAC T-3') for Calpastatin gene was used according to Schenkel *et al.* (2006). PCR amplification reactions were prepared in a total of 10 µl containing 5 µl PCR master mix (DreamTaq Green PCR Master Mix (2x)), 1 µl primer F and R (10 pmol/µl), 2 µl DDW, and 1 µl DNA template (50 ng/µl). The PCR amplification was conducted for 30 cycles by a thermalcycler machine (Eppendroft, USA) with a program of 94°C pre-denaturation for 2 min, 94°C denaturation for 30 sec, 58°C annealing for 45 sec, 72°C extension for 30 sec, 72°C final extension for 5 min. PCR product was visualized in 1% agarose, stained with ethidium bromide and checked the size by UV Transimullator (MUV21, MajorScience, USA) then documented by a camera.

2.4. Genotyping

Genotyping of Calpastatin gene used Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique with applying *Rsa*I enzyme restriction (GT^AC) for digestion. Five microliters of PCR product was added into mix reaction of 0.2 µl *Rsa*I enzyme (10 U/µl), 1 µl 10X Buffer and 3.8 µl aquabidest then incubated at 37°C for 4 hours. PCR-RFLP product was run in 2% agarose and visualized under UV light to determine genotype patterns.

2.5. Analysis

Genotype and allele frequencies were calculated directly according to Nei and Kumar (2000).

$$xii = \frac{\sum Nii}{N} \qquad \text{(a)}$$

$$xi = \frac{(2Ni + Nj)}{2N} \qquad \text{(b)}$$

where:

xii :Genotype frequency of Ai Ai
xi :Allele frequency of Ai
nii :number of genotype Ai Ai
Ni :number of allele Ai
Nj :number of allele Aj
N :total number of samples

Hardy-Weinberg equilibrium (HWE) was determined based on *Chi-Square* (χ^2) test (Hartl 1988).

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

where:
χ^2 :*Chi-Square* test value
O :the number of observed genotypes
E :the number of expected genotypes

3. Results

A 523 bp fragment of Calpastatin gene was successfully amplified (Figure 1). The PCR product was spanned from nt 18 to 516 according to GenBank AY008267.1 *Bos Taurus* (Schenkel *et al.* 2006). Genotyping of calpastatin was based on either presence or absence of the *Rsa*I restriction site (GT^AC), (Figure 2).

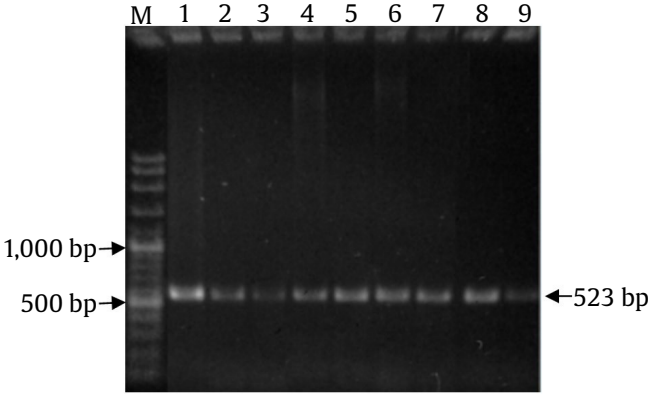

Figure 1. PCR products of calpastatin gene (523 bp) M=100 bp DNA ladder, 1-9=PCR products of Bali (1-4), Ongole (5-7), FH (8-9)

Three variant of genotype CAST gene of CC, CG, GG were found in three populations of cattle samples in Indonesia (Figure 3). CC genotype was performed in one fragment of 523 bp, GG genotype was in two fragments of 256 and 267 bp while GC genotype (heterozygote) was generated from three fragments of 256, 267, and 523 bp.

Genetic variation of Calpastatin gene was found in three populations of cattle samples. Ongole and Frisian Holstein (FH) cattle population were in genetic equilibrium based on Hardy-Weinberg while Bali cattle population was in genetic disequilibrium. Allele of G was dominant allele in cattle samples of Bali and Ongole. Frequency of C allele in Bali cattle population was found lower (0.12) than Ongole (0.50) and FH cattle (0.58) (Table 1).

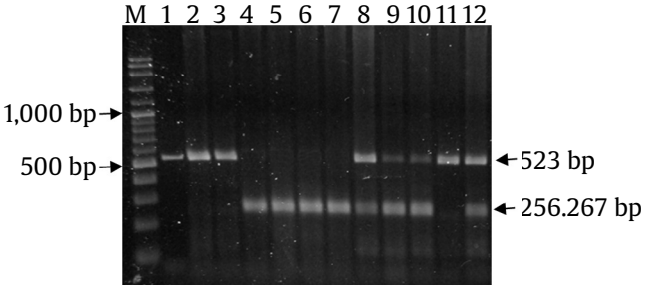

Figure 3. Genotypes of calpastatin gene M=100 bp DNA ladder, 1=PCR product (523 pb), 2, 3, 11=CC genotype, 4-7=GG genotype, 8-10, 12=GC genotype

Figure 2. Location of transversion G/C and *Rsa*I restriction site in calpastatin gene based on genbank AY008267.1 *Bos taurus* (Schenkel *et al.* 2006)

Table 1. Genotype and allele frequencies, hardy weinberg equilibrium of calpastatin gene in beef cattle (N=61)

Breeds	N		Genotype frequencies			Allele frequencies		χ^2 calculated
			GG	GC	CC	G	C	
Bali	21	Observed	18 (85.71%)	1 (4.77%)	2 (9.52%)	0.88	0.12	12.29
		Expected	16.2624	4.4352	0.3042			
Ongole	22	Observed	6 (27.27%)	10 (45.45%)	6 (27.27%)	0.50	0.50	0.99*
		Expected	5.5	11	5.5			
FH	18	Observed	4 (22.22%)	7 (38.88%)	7 (38.88%)	0.42	0.58	0.72*
		Expected	3.115	8.731	6.118			

χ^2 table$_{(0.05:2)}$ =5.99, *=genetic equilibrium (χ^2 table>χ^2 calculated)

4. Discussion

In the year of 70's Bali cattle has good performance in meat quality and even exported to overseas (Margawati *et al.* 2017). However, recently Bali cattle have performed varies from their original characters, such as decreased body weight (Hakim *et al.* 2008) and changing color pattern (Lindell 2013). We presumed that there must be in inbreeding influences subsequently affects to the meat quality. A molecular technology has facilitated to embark this molecular genetic research to trace meat quality of indigenous Bali cattle as has been famous in the past 70's. A PCR-RFLP is not a sophisticated method in present time however it is still helper tool to evaluate genetic variation in Calpastatin gene of Bali cattle.

This present study also involved the breeds of Ongole and Frisian Holsten (FH) which both have adapted very long in Indonesia to compare the main course of Bali cattle breed in genetic variation of Calpastatin gene. Genetic variation of Calpastatin gene was found in three involved cattle population of Bali, Ongole and FH. As statistical analysis, Ongole and Frisian Holstein (FH) cattle population were in genetic equilibrium based on Hardy-Weinberg while Bali cattle population was in genetic disequilibrium (Table 1). As stated by Beals *et al.* (1999), genetic disequilibrium is caused by several factors such as natural selection, mutation, nonrandom mating, genetic drift, and gene flow. Those condition might contribute to Bali cattle since Bali cattle is now distributed throughout of Indonesia and DNA Bali cattle samples in this study were collected from Breeding center. In the past of colonial era (Dutch), Bali cattle is restricted only in Bali Island to conserve the purity of Bali cattle. This regulation is still consistent running and now more expected pure Bali cattle conserved in Nusa Penida Island. However, the need of meat consumption is therefore Bali cattle are also reared outside of Bali cattle. This consideration of rearing Bali cattle (for the need of meat consumption) outside of Bali island is allowed by the regional regulation of Bali province (PERDA 2017).

Allele of G was dominant allele in Bali and Ongole cattle population compared to FH. It could be associated with origin of Bali cattle belongs to *Bos sondaicus* or *Bos javanicus* that distributed in Indonesia of Asia and Ongole cattle belongs to *Bos indicus*. It is different to FH cattle that distributed in Europe and originated from *Bos taurus* mostly less allele G (Schenkel *et al.* 2006; Pinto *et al.* 2010).

Frequency of C allele in Bali cattle population was found lower (0.12) than Ongole (0.50), and FH cattle (0.58) (Table 1). Bali cattle is an indigenous cattle in Indonesia and again the same reason with previous statement was due to originated from *Bos sondaicus* or *Bos javanicus* (Martojo 2012) and FH belongs to *Bos Taurus*. However, this finding of Bali cattle was similar to the previous study of Kök *et al.* (2013) that Turkish Grey Steppe (pure breed) have lower of allele C frequency when compared to Turkish Grey Steppe crossed breed (Turkish Grey Steppe x Brown Swiss). It seems that crossing to another breed from Europe (*Bos Taurus*) could increase Allele C. Another report of Savaşci and Atasoy (2016) found that Anatolian

Black and Eastern Anatolian Red both *Bos Taurus* origins have higher allele C frequency of 0.64 and 0.67 respectively.

Allele of C associated with meat tenderness in postmortem of cattle (*Bos taurus*) (Schenkel *et al.* 2006; Pinto *et al.* 2010). Meat quality was influenced by many factors such as breed, age, slaughter, pH, and feed (Guerrero *et al.* 2013). Crouse *et al.* (1989) reported that increase percentage of inheritance Brahman or Sahiwal (*Bos indicus*) in crossing cattle to *Bos taurus* caused decreasing of percentage tenderness scores and juiciness and increasing of shear value. Other researchers found that Brahman cattle have higher value of lean firmness and lower tenderness than Hereford (Wheeler *et al.* 1990). Tenderness of meat is related to calpastatin activity at postmortem. Increasing of calpastatin was followed by decreasing of calpain activity and caused decreasing proteolytic process in meat tenderness (Goll *et al.* 1998; Kemp *et al.* 2012). Ardicli *et al.* (2017) revealed that genotype CC has related significantly effect on final weight, fattening period, total weight gain, and average daily gain in Hanwoo cattle.

In this study, the Ongole cattle tends to have a potential source of lean meat quality in Indonesia. Finding of this recent study based on the genetic marker could be used to improve the genetic quality of local beef cattle in Indonesia.

5. Conclusions

Two alleles, C and G, of the Calpastatin gene were found in the local beef Bali cattle as well as in Ongole and Friesian Holstein, both of the latter two populations have also been adapted to conditions in Indonesia. The frequency of the C allele in Bali cattle was lower compared to that observed in Ongole and Friesian Holland. There is thus genetic variation in the Calpastatin gene that differ among the cattle populations sampled here. Improving meat quality in Bali cattle can be attempted through the introducing of Bali cattle with C allele in breeding program.

Conflict of interest

The authors declare that there is no conflict of interest.

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