A single nucleotide polymorphism of CAPN1 gene region 3’UTR in Bali cattle

Dairoh¹, Jakaria², M F Ulum³ and C Sumantri²

¹Post Graduate Student, Animal and Production Technology, Faculty of Animal Science, IPB University, Indonesia
²Department of Animal and Production Technology, Faculty of Animal Science, IPB University, Indonesia
³Department of Veterinary Clinic Reproduction and Pathology, Faculty of Veterinary Medicine, IPB University, Indonesia

E-mail: dairoh58@gmail.com

Abstract. Bali cattle (Bos javanicus), as Indonesian origin genetic resources, is domesticated from banteng (Bibos banteng). Bali cattle have the potential to be developed as producer of premium beef. Genes that have important role in meat quality are calcium-activated neutral protease genes, as known as calpains (CAPN). Calpains are classified as Ca²⁺ dependent intracellular cysteine proteases, including the ubiquitously expressed µ-calpain (CAPN1) and m-calpain (CAPN2). The purpose of this study was to analyze single nucleotide polymorphism (SNP) region 3’UTR CAPN1 gene in beef cattle. Polymorphism of CAPN1 gene was analyzed by direct DNA sequencing method in 42 Bali cattle that compared with 11 Belgian Blue, 7 Limousine, 12 Pasundan, and 12 Katingan. The result showed that CAPN1 gene has 7 polymorphic SNPs (g.15284 C>T, g.15347 T>G, g.15525 G>A, g.15674 C>T, g.15853 G>A, g.15905 G>A and g.15915 G>A) in Bali cattle. These SNPs that polymorphic in Bali cattle were monomorphics in Belgian Blue, Limousine, Pasundan and Katingan cattle. Only one SNP g.15853 G>A in Bali cattle was polymorphics in Belgian Blue. Deletion was detected that 8 nucleotides deletion (CTCCCTCC) occurred in Bali, Pasundan, and Katingan cattle at position g.15795 – g.15802, while Belgian Blue and Limousine cattle the deletion was not found.

1. Introduction

Bali cattle is known as Bos javanicus cattle breed which is an Indonesian origin cattle and it is as genetic resource that come from domestication of Banteng (Bibos banteng) [1]. Bali cattle have characteristics of physical form and genetic composition which have a high adaptability to marginalized environments [2]. Bali cattle has been recognized by the FAO as one of the cattle breeds in the world [3] spread throughout Indonesia. Bali cattle have considerable potential because they have a high degree of adaptation to marginalized environments [2], high reproductive power, are able to utilize low quality feed [4]. Furthermore, Bali cattle has high birth rates of 75-80% [5], carcass characteristic values of 56% [6] and potential to be developed as premium beef producer with better meat quality.

Calpain is the one of genes from 206 candidate genes that have important role in meat quality particularly meat tenderness [7]. Meat tenderness is determined by Calpains activity which is regulated by level of calcium content and calpastatin activity [8,9]. Calpain and calpastatin are two enzymes involved in calpain proteolytic system and have influenced in meat tenderness. Furthermore, calpain
and calpastatin genes are consist of six domains, which is main domain IV and VI of calpain to proteolysis activity is inhibited by amino acid from I to IV domain in calpastatin and also depend on calcium level in these genes. Calpastatin is located in cytosol with the result that calpain an inactive site because bound by calpastatin, however when calpain is translocated to membrane as result of high calcium level in intracellular and calpain in active site. Calpain1 (CAPN1) and calpain2 (CAPN2) are intracellular cysteine protease enzymes and are expressed ubiquitously [10,11]. The calpain1 (CAPN1) gene was detected in chromosome 29 [12,13] and consists of 21 exons and 20 introns [14]. Research of CAPN1 gene was once performed on Korean cattle at the region 3’UTR associated with marbling score and Bali cattle have not been widely studied in the 3’UTR region [15]. If a mutation found in 3’UTR region can influence the product of protein that expressed by translation regulation include translation efficiency, mRNA stability, and the level product of protein [16]. Therefore, the polymorphism of CAPN1 gene is potential for genetic markers influencing meat tenderness trait, particularly in Bali cattle. The aim of this study was to analyse the polymorphism of 3’UTR region CAPN1 gene in beef cattle by DNA sequencing methods.

2. Materials and methods

2.1. Materials
The number of five type of beef cattle (n=84 cattle) that used in this research were consist of Bali cattle (n=42 cattle) obtained from BPTU-HPT Denpasar, Bali Province and then was compared to Belgian Blue (n=11 cattle), Limousine (n=7 cattle), Pasundan (n=12 cattle), and Katingan cattle (n=12 cattle). Blood samples from 1.5 mL EDTA vacuum container was extracted by modified DNA extraction methods using the Geneaid Kit. Primer sequence design was used GenBank data base sequences (NCBI) with access code AH009246.3. Primer was determined by the length of the base using the Primer3 program. The predetermined primer was then analysed by the Multiple Primer Analyzer and Primer Stats program.

Table 1. Primary sequences the 3’UTR region of CAPN1 gene.

| Gene | Region | Primer Sequence | Temperature | Product (bp) |
|------|--------|-----------------|-------------|--------------|
| CAPN1 3’UTR | F : 5’–CTGCTCTCTATGCCCTCTCT–3’ | 61°C | 790 |
| | R : 5’–TCCAGAGACAAAAAGTGGGGT–3’ | | |

Note: F= forward; R= reverse

The amplification of the 3’UTR region of CAPN1 gene was carried out using DNA thermocycler AB system machine with the following protocol: 95°C pre-denaturation for 1 minutes was the first step and the second step was 35 cycles of 95°C for 15 seconds, 61°C annealing for 15 seconds, 72°C extension for 10 seconds, and also the third step of final extension at 72°C for 3 minutes. PCR reagent mixture was comprised of DNA concentration (2 µL), primer template of 0.3 µL forward and reverse respectively, 12.5 µL of MyTaq HS RedMix, and 9.9 µL of NFW. PCR product of 3’UTR region was electrophoresis through 1.5% of agarose gel. The DNA sequence of PCR amplicons were determined by the 1st Base laboratory service in Selangor, Malaysia with an ABI PRISM 96-capillary 3730 DNA Analyzer. The genotypes of the identified SNPs in the 84 beef cattle were determined by Molecular Evolutionary Genetic Analysis (MEGA10) program.

2.2. Genotype and allele frequencies
Analysis data including allele and genotype frequency, heterozygosity value, and chi-square test of Hardy-Weinberg equilibrium were calculated according to Nei and Kumar formulas and were performed by PopGene 1.32 software as follows [17]:
Allele frequencies: 
\[ x_i = \frac{(2n_{ii} + \sum_{i\neq j} n_{ij})}{2N} \]

Genotype frequencies: 
\[ x_i = \frac{n_{ii}}{N} \]

Where:
- \( x_i \) = Allele frequency of the \( i^{th} \) allele
- \( x_{ij} \) = Genotype frequency of the \( j^{th} \) genotype
- \( n_{ii} \) = Amounts of genotypes individuals with \( ii \) genotype
- \( n_{ij} \) = Amounts of genotypes individuals with \( ij \) genotype
- \( N \) = Amounts of samples

Heterozygous of observation (Ho) and heterozygous expectation (He) value were estimated by the Nei and Kumar formula as follows [17]:

\[ H_0 = \sum_{i \neq j} \frac{N_{ij}}{N} \quad H_e = 1 - \sum_{i=1}^{q} x_i^2 \]

Where:
- \( H_0 \) = Value of observed heterozygosity
- \( H_e \) = Value of expected heterozygosity
- \( N_{ij} \) = Amount of observed heterozygous samples
- \( N \) = Amounts of samples observed
- \( x_i \) = Allele frequency
- \( q \) = Amount of allelic

Chi-square test (\( \chi^2 \)) to estimate Hardy-Weinberg equilibrium of population was analysed by Allendorf et al (2010) formula as follows [17]:

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

Where:
- \( \chi^2 \) = Value of chi-square test
- \( O \) = The frequency of observed genotype value
- \( E \) = The frequency of expected genotype value

3. Results and discussion

3.1. Detection single nucleotide polymorphism of CAPN1 Gene
DNA samples from five type of beef cattle were successfully amplified using 61°C of annealing temperature at 3’UTR region of CAPN1 gene. Resulting band of PCR products matched the target, namely the 3’UTR fragment of the beef cattle CAPN1 gene measuring 790 bp. The result of PCR product in 3’UTR region can be seen in figure 1.
Annealing temperature in this study was compatible with PCR protocol standard that used by Patel et al (2018), namely 50°C – 62°C. The annealing temperature used in this study was different in several studies based on the reagent mixture [18]. Annealing temperature based on Muladno [19] can be calculated by the number of bases in the primer used.

![DNA amplification product of 3'UTR region of CAPN1 gene using 1.5% of gel agarose.](image)

**Figure 1.** DNA amplification product of 3'UTR region of CAPN1 gene using 1.5% of gel agarose.

DNA sequencing result of CAPN1 gene was presented in figure 2. In this result, the total of 7 new SNPs mutation was detected at 3'UTR region of CAPN1 gene, namely in the g.15284 C>T, g.15347 T>G, g.15674 C>T, g.15525 G>A, g.15853 G>A, g.15905 G>A, and g.15915 G>A bases position in Bali cattle. The alignment sequence of Bali cattle with another beef cattle using MEGAX software, the result showed that the SNPs found in Bali cattle was specific occur in Bali cattle sequence. Furthermore,
the CAPN1 gene at 3’UTR region of Bali, Pasundan, and Katingan cattle was found an 8 nucleotides deletion at the g.15795-g.15802 position with CTCCCTCC of bases sequence, while in Belgian Blue and Limousine did not found the 8 nucleotides deletion. This is also distinguished between Bali cattle with previous studies in the same region, namely the 3’UTR CAPN1 gene that there was no deletion in Hanwo cattle (Korean cattle) [15].

![Figure 3](image)

**Figure 3.** Visualization indel mutation of g.15795-g.15802 position at 3’UTR of CAPN1 gene

The 7 silent mutation on 3’UTR region in this study were substitution mutations consist of g.15284 C>T, g.15674 C>T, g.15525 G>A, g.15853 G>A, g.15905 G>A, and g.15915 G>A SNPs which all of them were transition mutation, however g.15347 T>G was transversion mutation in Bali cattle. Single Nucleotide polymorphism (SNP) is differences of nucleotide in sequence of genetic material that change the single nucleotide composition at a certain position, however single or more nucleotides insertion and deletion variation (Indel) is not considered as SNP [20]. Type of substitution mutation was divided into two types, namely transition and transversion mutation. Nucleotide change from purine to purine is known as transition mutation, however nucleotide change from purine to pyrimidine or pyrimidine to purine is known as transversion mutation [21]. Although the nucleotide changes in 3’UTR region was not change amino acid, the three untranslated regions contain polyadenylation and RNA binding site that have role important in post-transcriptional regulation. This regulation can influence translation efficiency, mRNA stability, and level of protein product [22].

### 3.2. Genetic diversity analyses among five cattle populations

The analysis data of genetic diversity parameter including allele and genotype frequency, heterozygosity value, and chi-square test ($\chi^2$) are shown in table 2. Allele and genotype frequency data was used to determine that the population was polymorphic or monomorphic. In this study, the g.15284 C>T, g.15674 C>T, g.15525 G>A, g.15853 G>A, g.15905 G>A, and g.15915 G>A SNPs mutation were polymorphic in Bali cattle, however in Belgian Blue, Limousine, Pasundan, and Katingan cattle were monomorphic. In addition, the g.15853 G>A SNP mutation was polymorphic in Bali cattle and Belgian Blue populations, however in Limousine, Pasundan, and Katingan cattle were monomorphic. The SNP mutation is mentioned polymorphic if the population has a number frequency of allele more than 0.01 in large population or 0.05 in small population [23]. All SNPs that found in this study was obtained two alleles and two or three genotypes in each population.

The heterozygosity value as well as observed and expected heterozygosity value is to estimate the genetic diversity level in the beef cattle population which is the diversity based on the 0-1 of heterozygous range value [24]. In this study, the heterozygosity value in each SNPs of 3’UTR region was from 0.048 to 0.286. The higher of heterozygosity value was occurred in the g.15284 C>T of SNP mutation and the smaller heterozygosity value was in the g.15915 G>A of SNP mutation, it means that the genetic diversity of g.15915 G>A SNP mutation was lower. The chi-square test data is to estimate whether the beef cattle population is mentioned within Hardy-Weinberg equilibrium. In this case, four of the SNPs g.15284 C>T, g.15525 G>A, g.15853 G>A, and g.15905 G>A were in Hardy-Weinberg equilibrium, it was shown that the chi-square test count was smaller than chi-square table ($\alpha$ 0.05 df 1: 3.84). Gene equilibrium is occurred in the absence of mutation, selection, migration, and genetic drift.
[25]. The 3’UTR of g.15347 G>T, g.15674 C>T, and g.15915 of SNPs mutation were in disequilibrium, it was means that the population in these SNPs not accordance with Hardy-Weinberg law.

Table 2. Genotype frequency, allele frequencies, heterozygosity, and chi-square test ($\chi^2$) CAPN1 gene value at various locus in five types of Bali, Belgian Blue, Limousine, Pasundan, and Katingan populations.

| SNP   | Population | N   | Genotypic frequency | Allelic frequency | Ho  | He  | $\chi^2$ |
|-------|------------|-----|---------------------|-------------------|-----|-----|----------|
|       |            |     | CC      | CT      | TT | C  | T  |        |
| g.15284 | Bali       | 42  | 0.71  | 0.29   | 0.00 | 0.86 | 0.14 | 0.286 | 0.248 | 1.059<sup>ns</sup> |
| C>T   | Belgian Blue | 11  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Limousine  | 7   | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Pasundan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Katingan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       |            |     |        |        |     | TT | T  |        |
| g.15347 | Bali       | 42  | 0.05  | 0.07   | 0.88 | 0.08 | 0.92 | 0.071 | 0.155 | 14.029<sup>ns</sup> |
| T>G   | Belgian Blue | 11  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Limousine  | 7   | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Pasundan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Katingan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       |            |     |        |        |     | G  | A  |        |
| g.15525 | Bali       | 42  | 0.02  | 0.26   | 0.71 | 0.15 | 0.85 | 0.262 | 0.265 | 0.005<sup>ns</sup> |
| G>A   | Belgian Blue | 11  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Limousine  | 7   | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Pasundan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Katingan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       |            |     |        |        |     | C  | T  |        |
| g.15674 | Bali       | 42  | 0.05  | 0.00   | 0.95 | 0.05 | 0.95 | 0.000 | 0.092 | 55.359<sup>ns</sup> |
| C>T   | Belgian Blue | 11  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Limousine  | 7   | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Pasundan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Katingan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       |            |     |        |        |     | G  | A  |        |
| g.15853 | Bali       | 42  | 0.81  | 0.19   | 0.00 | 0.90 | 0.10 | 0.191 | 0.174 | 0.403<sup>ns</sup> |
| G>A   | Belgian Blue | 11  | 0.81  | 0.09   | 0.00 | 0.95 | 0.05 | 0.100 | 0.100 | 0.000<sup>ns</sup> |
|       | Limousine  | 7   | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Pasundan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Katingan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       |            |     |        |        |     | GA  | A  |        |
| g.15905 | Bali       | 42  | 0.98  | 0.02   | 0.00 | 0.99 | 0.01 | 0.238 | 0.238 | 1.000<sup>ns</sup> |
| G>A   | Belgian Blue | 11  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Limousine  | 7   | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Pasundan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Katingan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       |            |     |        |        |     | GA  | A  |        |
| g.15915 | Bali       | 42  | 0.93  | 0.05   | 0.02 | 0.95 | 0.05 | 0.048 | 0.092 | 12.821<sup>ns</sup> |
| G>A   | Belgian Blue | 11  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Limousine  | 7   | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Pasundan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |
|       | Katingan   | 12  | 1.00  | 0.00   | 0.00 | 1.00 | 0.00 | 0.000 | 0.000 |               |

Note: SNP = single nucleotide polymorphism; N = number of populations; <sup>ns</sup> = non-significant at P>0.05 or $\chi^2$ count < $\chi^2$ table (α 0.05; df 1 = 3.84); nc = not counted

Discovery SNP mutation of the 3’UTR region CAPN1 gene was limited studied in beef cattle. The information of 3’UTR SNP had been previously studied by Juszczuk-Kubiak [26] in Charolaise,
Simmental, Fresian, Polish Red, Hereford and Limousine. Furthermore, Cheong et al (2008) also showed from the result of his studied that the polymorphism of 3’UTR region CAPN1 gene was significantly had association with marbling score in Hanwoo cattle (Korean cattle) [15]. From these finding in this study, it is possible that the discovery SNP which found in 3’UTR of CPAN1 gene was related with meat quality in Bali cattle. Therefore, the future study is suggested to validate whether traits that was influenced by the polymorphism of CAPN1 gene, particularly at 3’UTR region of Bali cattle.

4. Conclusion
This result confirmed that the total of 7 SNPs mutations had been found in the 3’UTR of CAPN1 gene in Bali cattle. All these SNPs was polymorphic in Bali cattle but was monomorphic in another beef cattle of this study. Four of seven SNPs include g.15284 C>T, g.15525 G>A, g.14853 G>A, and g.15905 G>A were in Hardy-Weinberg equilibrium in Bali cattle.

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