# Association of SNP g.232 G>T Calpain Gene with Growth and Live Meat Quality Prediction using Ultrasound Images in Bali Cattle

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**ABSTRAK**

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Sapi Bali (*Bos javanicus*) adalah ternak asli Indonesia hasil domestikasi dari banteng (*Bibos banteng*). Gen yang memiliki peran penting terhadap kualitas daging adalah proteinase sistein netral yang diaktivasi oleh kalsium, dikenal sebagai calpain (CAPN). Tujuan dari penelitian ini adalah untuk menganalisis keragaman gen calpain dan hubungannya dengan sifat pertumbuhan dan kualitas daging dalam bentuk tebal *longissimus dorsi* (TLD), tebal lemak punggung (TLP), skor marbling, dan presentase lemak intramuskular (PLIM) pada sapi Bali. Keragaman gen CAPN1 dianalisis melalui PCR-RFLP menggunakan enzim restriksi BglII pada sapi Bali (n=52 ekor). Citra ultrasonografi otot longissimus dorsi diambil dengan posisi transversal dan longitudinal diantara tulang vertebrae torakalis ke 12-13 dan dianalisis dengan perangkat lunak Image-J NIH. Hasil penelitian menunjukkan bahwa SNP g.232 G>T gen CAPN1 bersifat polimorfik pada sapi Bali. SNP g.232 G>T gen CAPN1 pada sapi Bali memiliki variasi tinggi yang ditunjukkan dengan nilai heterozigositas 0.48 dan berada dalam keseimbangan Hardy-Weinberg. Keragaman SNP g.232 G>T berasosiasi secara signifikan (P<0.05) dengan bobot badan 730 hari, skor marbling (SM), dan presentase lemak intramuskular (PLIM) pada sapi Bali. Disimpulkan bahwa gen CAPN1 pada sapi Bali adalah kandidat sebagai Marker Assisted Selection (MAS) yang memiliki pengaruh terhadap bobot badan 730 hari, skor marbling, dan presentase lemak intramuskular.

**Kata Kunci**: Sapi Bali, Gen calpain, Pertumbuhan, Kualitas daging, Ultrasonografi

**ABSTRACT**

Dairoh, Jakaria, Ulum MF, Ishak ABL, Sumantri C. 2020. Association of SNP g.232 G>T calpain gene with growth and live meat quality prediction using ultrasound images in Bali Cattle. JITV 26(2): 49-56. DOI: http://dx.doi.org/10.14334/jitv.v26i2.2701.

Bali cattle (*Bos javanicus*) are native Indonesian cattle, domesticated from banteng (*Bibos banteng*). Genes that have an important role in meat quality are calcium-activated neutral protease genes, known as calpains (CAPN). The objective of this study was to evaluate the polymophisms of calpain gene SNP g.232 G>T by PCR-RFLP technique and its influence on growth trait and meat quality of Bali cattle detected by ultrasound imaging of *longissimus dorsi* thickness (LDT), back fat thickness (BFT), marbling score (MS), and intramuscular fat percentage (PIMF). The polymorphisms of CAPN1 gene were analyzed by PCR-RFLP using BglII restriction enzyme (n=52 cattle). The ultrasound images of longissimus dorsi muscle were carried out transversally and longitudinal between $12^{th}$ -$13^{th}$ thoracic vertebrae then analyzed by Image-J NIH software. Result showed that SNP g.232 G>T of CAPN1 gene was polymorphic in Bali cattle. SNP g.232 G>T of CAPN1 gene in Bali cattle has higher diversity which was showed of 0.48 heterozygosity value and was in Hardy-Weinberg equilibrium. The polymorphisms of SNP g.232 G>T was associated significantly (P<0.05) with bodyweight at 730 days, marbling score (MS), and intramuscular fat percentage (PIMF). It suggests that the CAPN1 gene in Bali cattle is a candidate for Marker Assisted Selection (MAS), which influences body weight at 730 days, marbling score, and percentage of intramuscular fat.

**Key Words**: Bali cattle, Calpain gene, Growth, Meat quality, Ultrasound

## INTRODUCTION

In 2020, a total of national meat production was dominated by 59% of chicken, the contribution of beef cattle only 16% into the total national meat production. National beef demand can only be fulfilled by 73.94% of domestic production, and 26.06% of the deficiency is fulfilled by imports (OECD-FAO 2020). Efforts to increase beef production through the breeding program are still focused on growth, while the increasing

demand for meat requires not only quantity but also quality parameters for consumers. The selection of the right beef cattle for slaughter significantly affects meat quality. Therefore rapid method of prediction of optimal fattening period length and measurement to determine the slaughter value in live animal are required (Pogorzelska-Przybylek et al. 2015). Ultrasound imagery is a rapid and non-invasive tool which believed to be an accurate predictor for fat and muscle deposition in livestock (Silva & Cadavez 2012). Ultrasound technology was also used to predict the carcass of beef at 12-13$^{th}$ fat and *longissimus dorsi* thickness (Jakaria et al. 2017). Ultrasonic rump fat and back fat thickness showed a high correlation of 0.64 (Bonin et al. 2015). Ultrasound measurements, including IMF percentage, have shown to be a valuable method for assessing carcass quality characteristics (Nogalski et al. 2018). Non-invasive technology is a well-established method used to assess the quality of live meat prior to slaughter in the cattle breeding program for native cattle.

Bali cattle (*Bos javanicus*), as a genetic resource for native cattle, come from the domestication of banteng (*Bibos banteng*) (Martojo 2012). Bali cattle also have been recognized by the FAO as one of the cattle breeds in the world in 2003 (Directorate General of Livestock Service 2003). Bali cattle have several superiorities, including fertility rates (pregnancy) range from 80% to 90%, birth rates of 75-85% (Wawo 2018) and carcass values of 56%, and good meat quality (Hafid et al. 2019). Bali cattle have the potential to be selected as a premium beef by the influence candidate gene related to meat quality which is Calpains gene (CAPNs). By using the molecular approach, the CAPNs gene can be evaluated for their nucleotide sequence profile. Calpain plays an important role in postmortem proteolysis, which can degrade the myofibril and influence meat tenderness by the proteolytic system (Coria et al. 2018). Calpain activity is regulated by calcium levels and calpastatin as a specific inhibitor. Calpain and calpastatin are two enzymes involved in the process of the proteolytic calpain system. The Calpastatin inhibits the calpain activity in low calcium levels of cytosol (Lian et al. 2013). Muscle growth in meat has a negative effect on the proteolysis activity of calpain, one of which is the muscle growth controlled by the myostatin gene (MSTN). Hypertrophy usually occurs in connective tissue. For example, double muscle is due to deletion of the MSTN gene, which causes a decrease of protein degradation and results in tough meat (Koohmaraie et al. 2002).

The influence of CAPN on meat quality, including in terms of tenderness, muscle fiber characteristic, and fatty acid, has been reported in Japanese quail (Işık 2019), sheep (Zhang et al. 2016), and pork (Lee et al. 2012; Lim et al. 2014). It was also reported in the Korean cattle, namely Hanwoo that calpain has many important roles in marbling score and intramuscular fat content, particularly in untranslated regions (Cheong et al. 2008). If a mutation variant takes place in calpain untranslated region, it can be influenced by the level of the protein product from translation and stabilization of RNA expression (Steri et al. 2018). This process is related to meat quality. The influence of genetic variants in the untranslated region has also been reported: to have an important role in the regulation of the post-transcription process (Araujo et al. 2012), initiates modulator translation (Dacheux et al. 2017), and translation regulation (Mayr 2017). Although the influence of the calpain gene has been widely investigated in the populations of *B. taurus*, *B. indicus*, and *B. taurus* x *B. indicus*, however, it is less studied in the untranslated region of Bali cattle (*Bos javanicus*) up to the present.

Therefore, the presence of calpain genes is necessary to be studied using as a potential candidate of genetic markers in Bali cattle for the selection program. The objective of this study was to evaluate the influence of SNP g.232 G>T calpain gene by PCR-RFLP technique on growth and meat quality traits in Bali cattle.

## MATERIALS AND METHODS

### Blood sampling

A total of 52 Bali cattle blood samples were used in this study, which was obtained from BPTU-HPT Denpasar of Bali Province. Those blood samples were taken from the *jugular vein* and collected using venoject tubes containing 1.5 ml EDTA. Extraction of DNA was carried out by DNA extraction kits (Geneaid) at the Animal Molecular Genetic Laboratory of the Faculty of Animal Science of IPB University.

### Phenotypic data

The phenotypic data observed was including *longissimus dorsi* thickness and back fat thickness (carcass characteristics), marbling score and percentage of intramuscular fat (meat characteristics) measured using a 6.5 MHz transducer of ultrasound images at 12-13$^{th}$ rib position (Ulum et al. 2014). The ultrasound image result was stored in JPEG format and performed by Image-J NIH software (ImageJ, NIH, USA) (Figure 1). The determination of the marbling score (MS) was based on the AUSTRALIAN MEAT and MSA (http://www.wagyu.org.au/marbling/).

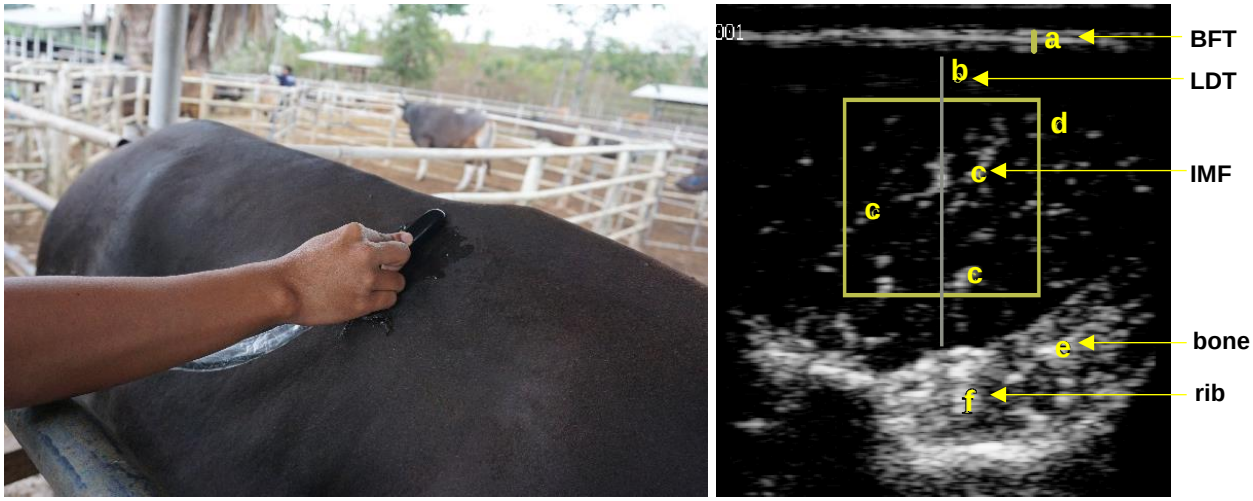


**Figure 1.** The position of muscle ultrasound measurement at 12-13th rib orientation point (left) and the image analyzed by using Image-J NIH software (right), (line a = back fat thickness (BFT); line b = longissimus dorsi thickness (LDT); c = intramuscular fat content (IMF), rectangle d = measurement area IMF 30 mm x 30 mm; e = bone; f = rib)

### Primer design and DNA amplification

The primer sequences of the CAPN1 gene based on Jakaria et al. (2020) were previously designed at the 5'UTR region using GenBank database sequence (NCBI) with access code AH009246.3. The primer sequences included forward and reverse as follows F: 5'-CCCTTCCCACCCAGATAGG-3', R: 5'-CCTGGAGACCGTGAGGAAC-3' and the PCR product was 478 bp. The amplification of the CAPN1 gene at 5'UTR region were conducted using thermocycler AB System machine with PCR condition of pre-denaturation at 95°C for 1 minute, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 57°C for 15 seconds, extension at 72°C for 10 seconds, and a final extension step at 72°C for 3 minutes. PCR product was visualized through 1.5% agarose gel and was observed using UV Transilluminator (Biorad™, California, USA).

### PCR-RFLP and genotyping

PCR product was incubated at 37°C for 4 hours with *Bgl*II restriction endonuclease enzyme for digesting SNP g.232 G>T at 5'UTR region. The sample products were then verified using electrophoresis through 2% agarose gel with PeqGreen staining for genotyping. Identification of the genotype was determined by band pattern visualization.

### Data analysis

Genetic diversity parameters of allele and genotype frequencies, heterozygosity, and chi-square were calculated by direct counting, namely PopGen 1.32 program. The frequency of alleles and genotypes were calculated using the Nei & Kumar (2000) formula as follows:

$$X_i = \frac{(2n_{ii} + \sum_{i \neq j} n_{ij})}{2N} \qquad X_i = \frac{n_{ii}}{N}$$

Where $\mathbf{x_i}$ is allele frequency, $\mathbf{x_{ii}}$ is genotype frequency, $n_{ii}$ is the number of individuals with genotypes ***ii***, $n_{ij}$ is the number of individuals with genotypes ***ij***; and N is the sample number of individuals.

Furthermore, Genetic diversity is calculated using the Nei & Kumar (2000) formula using observed heterozygosity ($H_o$) and expected heterozygosity ($H_e$) value as follows:

$$H_O = \sum_{i \neq j} \frac{N_{1ij}}{N} \qquad H_e = 1 - \sum_{i=1}^{q} x_i^2$$

Where $H_o$ is heterozygosity observation value, $N_{1ij}$ is the number of individuals with heterozygous, N is the observed number of individuals, $H_e$ is heterozygosity expectations value, $x_i$ is frequency of allele, and q is the alleles number.

Hardy-Weinberg equilibrium is analyzed by chi-Square ($\chi^2$) according to Nei & Kumar (2000) as follow:

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

Where $x^2$ is *chi*- Square, O is observed value, and E is expected value.

The association between each of SNPs in CAPN1 gene with carcass and meat characteristic in Bali cattle were performed using SAS 9.4 software by General Linear Model (GLM) procedure (SAS Inst, Inc, Cary, NC). Tukey Multiple Comparison Test ANOVA was compared for the least means square value of

genotypes. The mathematical of GLM model as follows:

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where $Y_{ij}$ is observation of phenotypic, μ is overall mean, $G_i$ is effect of genotypes, and $e_{ij}$ is random error.

Meat characteristic and growth traits data were corrected based on age and maintenance system before calculating association using the formula according to Salamena & Papilaja (2010) as follows:

$$\text{Xi corrected} = \left[\frac{\bar{X}_{\text{standard}}}{\bar{X}_{\text{observation}}}\right] \text{ x X observation value i}$$

Where $X_{i\ \text{corrected}}$ is corrected data I, $\bar{X}_{\text{standard}}$ is standard group average, $\bar{X}_{\text{observation}}$ is observation group average, and $X_{\text{observation value i}}$ is observation value data i

Live body weight of Bali cattle was corrected on age and maintenance using formula according to Hardjosubroto (1994) as follows:

$$\text{BWDG} = \left[\frac{\text{Bi-B0}}{\text{Age}}\right] \qquad B_{205} = \left[\frac{\text{Bi-B0}}{\text{Age}} \text{x } 205\right] + \text{B0}$$

$$B_{365} = \left[\frac{\text{Bi-B0}}{\text{Age}} \text{x } 365\right] + \text{B0} \qquad B_{730} = \left[\frac{\text{Bi-B0}}{\text{Age}} \text{x } 730\right] + \text{B0}$$

Where BWDG is body weight daily gain, Bi is body weight at weighing data I, B0 is birth weight, $B_{205}$ is body weight at 205 days, $B_{365}$ is body weight at 365 days, and $B_{730}$ is body weight at 730 days.

## RESULTS AND DISCUSSION

### Polymorphisms of SNP g.232 G>T 5'UTR Region

The 5'UTR region was successfully amplified at 57 °C, and the PCR product was 478 bp (Figure 2A). The polymorphisms of SNP g.232 G>T were analyzed using the PCR-RFLP. Three distinct-genotype patterns were found in Bali cattle (Figure 2B). *Bgl*II restriction digestion result was in fragment length of 478 bp for genotype GG, and 478 bp, 354 bp, 124 bp for heterozygotes GT, in addition, 354 bp, 124 bp for genotype TT (Figure 2B). The GG genotype is a GenBank reference genotype representing the wild-type allele, whereas the TT genotype represents a mutant genotype with two mutant alleles (National Center for Biotechnology Information 2021). This corresponds to the Ensembl SNP reference with the location code rs44090872 or c.-11 G>T by access code ENSBTAG00000010230 (EnsemblGenomes 2021).

A previous study has been reported that the 5'UTR region of CAPN1 gene was polymorphic in Bali cattle nevertheless quite low nucleotide diversities (Pi) of 0.00632 (Jakaria et al. 2020). Previously, the role of CAPN gene has been reported that the u-calpain is the enzyme proteolysis, contributes to meat tenderness (Avilés et al. 2013; Lozano et al. 2016). The coding region of CAPN1 gene polymorphisms association with meat quality has been widely reported in cattle. Chung et al. (2014) revealed that the CAPN1 gene had a significant effect on meat tenderness in Hanwoo cattle. Furthermore, the polymorphism of CAPN1 gene has been reported to be associated with fatty acid and amino acid in Yanbian Yellow cattle (Xin et al. 2010). However, there was no information about the association of 5'UTR with meat quality in Bali cattle. According to Sihite et al. (2019), Bali cattle with the GG genotype have the highest value of live weight and average daily gain than the GT and TT genotypes.

### Genotype and allele frequencies and heterozygosity value of 5'UTR region

Genetic diversity value in a population is stated as a parameter for studying population and evolutionary genetics. The variation of the population can be seen from allele and genotype frequencies. The allele

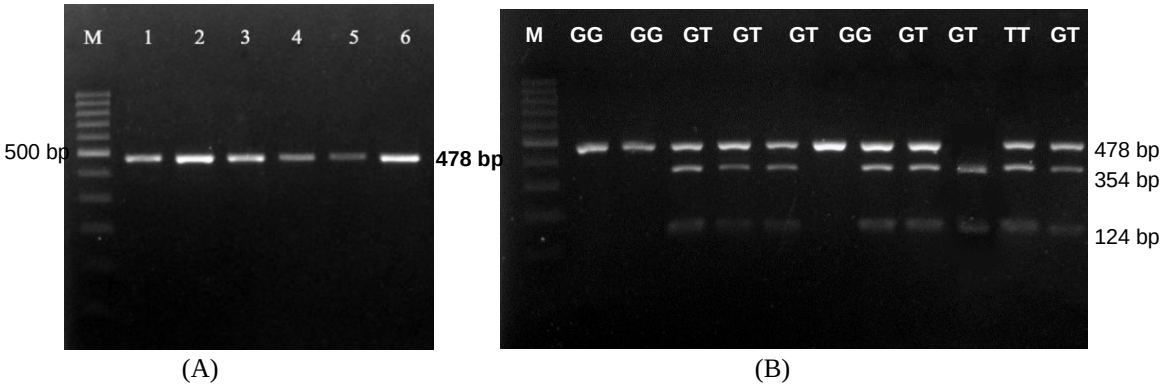


**Figure 2.** PCR amplification and RFLP genotyping of the polymorphism within CPAN1 gene's 5'UTR region in Bali cattle. (A) electrophoresis 1.5% gel agarose. (B**)** electrophoresis 2% gel agarose showing three PCR-RFLP genotypes pattern. M = 100 bp; line 1-6 = samples

**Table 1.** Allele frequency, genotype frequency, heterozygosity, and chi-square test SNP g.232 G>T Calpain gene at 5'UTR region in Bali cattle

| Breeds | N | Genotype Frequency | | | Allele Frequency | | Ho | He | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|
| | | GG | GT | TT | G | T | | | |
| Bali | 52 | 0.46 | 0.48 | 0.06 | 0.70 | 0.30 | 0.481 | 0.423 | $1.016^{ns}$ |

Note: N = total sample; ns = not significant P>0.05 or $\chi^2$ value < $\chi^2$ (α 0.05 df 1 = 3.84)

**Table 2**. Association analysis of SNP g.232 G>T polymorphism with meat quality and growth traits in Bali cattle

| Traits | Genotype | | | P-value |
|---|---|---|---|---|
| | GG (n=23) | GT (n=25) | TT (n=1) | |
| Weaning weight (kg) | 92.67 ± 22.55 | 86.82 ± 14.79 | 84.00 ± 0.00 | 0.544 |
| Body weight at 365 days (kg) | 142.30 ± 48.80 | 120.32 ± 29.68 | 105.00 ± 0.00 | 0.145 |
| Body weight at 730 days (kg) | $278.90 \pm 77.10^{a}$ | $230.20 \pm 54.70^{b}$ | $295.00 \pm 0.00^{ab}$ | 0.042 |
| Average daily gain (kg) | 0.33 ± 0.08 | 0.29 ± 0.06 | 0.28 ± 0.00 | 0.138 |
| | GG (n=24) | GT (n=25) | TT (n=3) | |
| *Longissimus dorsi* thickness (mm) | 52.95 ± 4.88 | 51.44 ± 4.47 | 53.01 ± 7.30 | 0.527 |
| Back fat thickness (mm) | 1.91 ± 0.35 | 1.68 ± 0.31 | 1.90 ± 0.31 | 0.058 |
| Marbling score | $1.63 \pm 0.69^{a}$ | $1.18 \pm 0.26^{b}$ | $1.56 \pm 0.71^{ab}$ | 0.024 |
| Intramuscular fat (%) | $3.01 \pm 1.57^{a}$ | $1.79 \pm 0.85^{b}$ | $2.68 \pm 1.68^{ab}$ | 0.006 |

[a-b] Different letters indicate a significant difference between genotypes, P<0.05

frequency, genotype frequency, and heterozygosity value of SNP g.232 G>T polymorphisms in Bali cattle are presented in Table 1. Two alleles and three genotypes of SNP g.232 G>T were found in Bali cattle. Approximately half of the Bali cattle were heterozygous (48%) in SNP g.232 G>T polymorphism of the 5'UTR region. It was assumed that the Calpain/*Bgl*II fragment was polymorphic in Bali cattle. If an allele frequency is 0.99 in a large population or 0.95 in a small population, it is confirmed to be polymorphic (Allendorf et al. 2013).

Based on the chi-square test, Bali cattle were in Hardy-Weinberg equilibrium (P>0.05). Equilibrium of this breed was also described by a comparison of observed and expected heterozygosity values in this study. The observed heterozygosity was higher than expected heterozygosity, and it is suggested that the population was in random mating effect (Sharma et al. 2016). The heterozygosity of Bali cattle was high, about 0.481, which means that Bali cattle have plenty of genetic diversity. Sheriff & Alemayehu (2018) stated that the population with high heterozygosity means lots of genetic variability, of about ≥50%. From these findings, it can be determined that polymorphism of SNP g.232 G>T in Bali cattle was still under the Hardy-Weinberg rule, which there is no mutation, migration, selection, and also random mating (Khan et al. 2018).

**Association of genotype with growth and meat quality traits**

Table 2 is presenting the mean and standard deviation values for each growth and meat quality of Bali cattle. The result of association analysis between 5'UTR of calpain gene and growth traits showed that the SNP g.232 G>T polymorphisms was associated with body weight at 730 days. Bali cattle with the GG genotype had the highest body weight at 730 days compared to the GT genotype. Sihite et al. (2019) showed that the CAPN1 gene was associated with birth weight and average daily gain, which differs from this present study in terms of the number of samples used. These findings suggested that SNP g.232 G>T polymorphisms potentially be used as the candidate of Marker Assisted Selection (MAS) for selection criteria for growth trait. This finding is supported by Zhang & Li (2011) showed that the Calpain gene has a strong association with bodyweight, weaning weight, and birth weight in Nanyang cattle. Furthermore, Pintos & Corva (2011) stated that one or more genes in bovine

chromosome 29, including the calpain gene, were involved in growth regulation.

The polymorphisms of SNP g.232 G>T at 5'UTR region was associated with marbling score and intramuscular fat percentage in Bali cattle (P<0.05). It showed a strong impact on the different genotypes of these traits. The highest marbling score and intramuscular fat percentage were found in Bali cattle with GG genotypes compared to the TT and GT genotypes. The degree of marbling is generally considered contributing to the sensory quality, in particular the tenderness of cooked meat (Wang et al. 2016). Newlacil et al. (2013) revealed that the marbling score of Charolais and Limousin were 1.54 and 1.27 lower than the marbling score of this study (1.63). This result indicated that the marbling score increase with increasing intramuscular fat content. Lee & Choi (2019) stated that the marbling score and intramuscular fat content have strong correlation ranges from 0.80 up to 0.88 in Hanwoo steer.

Meat quality prediction can be carried out by the actual technique (real-time) or using non-invasive technology as ultrasound imaging. In an actual prediction, male Bali cattle have carcass quality i.e., back fat thickness 3.08 mm, carcass percentage 53.33%, and marbling score of 2.65 (Suryanto et al. 2014). Nellore cattle with a backfat thickness <3 mm had a marbling score of 3.39, while back fat thickness >6 mm had marbling score 3.50 (Malheiros et al. 2015). Hanwoo cattle back fat thickness was 10 mm with marbling score 9.55 (Moon et al. 2003). These results indicate that different breeds can perform different meat quality. Ultrasound imaging is an efficient technology to predict carcass and meat measurement, potentially used in the breeding program. Robinson et al. (1992) stated that the accuracy of ultrasound was obtained from the correlation value with carcass measurement, i.e., rump thickness 92%, rib fat 90%, and longissimus muscle 87%. The backfat thickness and rump fat thickness of Nellore cattle using ultrasound imaging technology were 0.44 and 0.47, respectively (Bonin et al. 2015). Australian Angus cattle heritability value of intramuscular fat was 0.62, and marbling score was 0.46 (Duff et al. 2018)

The association of polymorphism in the UTR region clearly shows a strong association with both meat quality and growth trait (P<0.05) in Bali cattle. The UTR region influences gene expression by affecting mRNA stability and translation efficiency (Gu et al. 2014). There is evidence by Schuster & Hsieh (2019) that two differences of 5'UTR region alternative can increase the expression of the cancer tumor suppressor gene through a transition process. This transition is from the shorter of 5'UTR expression as efficient translation to the longer 5'UTR expression, which contains the secondary structure, and uORF result greatly hinders translation. It is possible that mutation SNPs in the UTR region could affect to the stability of the mRNA and translation process and resulting in a changed function (Juszczuk-Kubiak et al. 2010). Cheong et al. (2008) stated that the polymorphism in the 3'UTR region of the CAPN1 gene had a strong association (P-value: 0.0007) with the marbling score in Hanwoo cattle. Therefore, the polymorphism of the calpain gene in the UTR region is expected in future investigation as a genetic marker in Bali cattle associated with meat quality and growth traits.

## CONCLUSION

This study confirmed that the SNP g.232 G>T of calpain gene in Bali cattle was one of the candidate genes for marbling score and percentage of intramuscular fat, which showed a strong association (P<0.0.5). Furthermore, the SNP g.232 G>T polymorphism also has an association with the growth trait of body weight at 730 days. The information of significantly associated SNP g.232 G>T could be used as a Marker Assisted Selection (MAS) candidate in Bali cattle.

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