# Genetic Variation in the Calpastatin Gene and its Association with Growth Traits in Batur Sheep

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**Submission date:** 08-Apr-2023 07:46AM (UTC+0700)
**Submission ID:** 2058711412
**File name:** makalah_6..pdf (739.18K)
**Word count:** 5056
**Character count:** 23874

KnE Life Sciences

ICASI
3rd International Conference on Advance & Scientific Innovation
ICASI – Life Sciences Chapter
Volume 2022

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# Genetic Variation in the Calpastatin Gene and its Association with Growth Traits in Batur Sheep

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**Abstract.**

The present study aimed to investigate the association of the CAST genotype and growth traits in Batur sheep. Batur lambs were reared under an intensive feeding system. Bodyweight is measured monthly after weaning until six months of age. Blood representing thirty head were collected, genomic DNA was extracted as samples, and then 200 µl of whole blood samples were used. Specific primers were designed to amplify the CAST gene, samples were sequenced, then the researchers used the BioEdit program to identify any mutation. Calculation of genotypes, gene and allele frequencies, heterozygosities, and Chi-square test was performed. The analysis revealed a total of sixteen polymorphic sites in the CAST coding region. There are four alleles observed (A, G, C, and T), trans-versions at c.92T<G and c.285G>T loci, and transitions at c.214G>A, c.280G>A, c.301C<T, and c.371A>G. One individual disrupted the reading frame in the whole CAST sequenced. The genotype frequency analysis showed the highest predominance of the (TT, GG, CC, and AA) genotypes with frequencies (66.7 %, 62.9 %, 59.3 %, 66.7 %, and 55.6 %) as homozygous. In contrast, the heterozygous (TG, GA, GT, CT, and AG) genotypes were present at lower frequencies (29.6 %, 33.3 %, 37 %, 29.6 %, and 37 %), respectively with four difference alleles (T, G, C, and A). Post-weaning bodyweight till 6-month age of Batur sheep for the heterozygous genotypes at loci (c.92T<G, c.214G>A, c.280G>A, and c.301C<T) was slightly more massive than those carrying homozygous wild-type genotypes (c.285G>T and c.371A>G) with no significant differences ($p > 0.5$). The lambs with the heterozygous genotype had a significantly higher muscle percentage as a whole, compared to the other genotypes. Polymorphic sites were present in Batur sheep for the first time about the association with the CAST gene. However, the CAST gene might not be a useful marker for developing future selection programs in Batur sheep unless further investigation of the CAST gene and its interactions with other genes involving muscle growth and carcass traits are analyzed through association studies at a large scale.

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**Published**: 13 Sepetmber 2022

Publishing services provided by Knowledge E



Selection and Peer-review under the responsibility of the ICASI Conference Committee.

OPEN ACCESS

**Keywords:** genetic variation, CAST gene, association, Batur sheep, growth traits

**How to cite this article:** H I H Haren*, D Purwantini, M Y Sumaryadi, A Setyaningrum, A Susanto and M Pangestu , (2022), "Genetic Variation in the Calpastatin Gene and its Association with Growth Traits in Batur Sheep" in *3rd International Conference on Advance & Scientific Innovation ICASI – Life Sciences Chapter*, KnE Life Sciences, pages 420–431. DOI 10.18502/kls.v0i0.11827

## 1. Introduction

CAST is encoding the Calpastatin gene, which is located on chromosome 5, a chromosome including one of the three genomic regions associated with fat deposition in sheep [1]. CAST gene contains 36 exons and 35 introns in sheep [2]. Exon 6 is the largest exon of the ovine CAST gene [3]. Calpastatin is the only currently known endogenous specific inhibitor of Calpains 1 and 2 that does not affect other proteases. Calpastatin is present in all cell compartments of various tissues [4]. The CAST gene inhibits Calpain by preventing the Calpain proteolytic activation, membrane binding, and the expression of catalytic activity [5]. Calpastatin is involved in various physiological processes in the body, such as the regulation of protein turnover, and growth (Goll et al., 2003[6], fusion [7], and myoblast migration [8]. The calpain-calpastatin system influences many important processes including muscle development and growth as well as post-mortem meat tenderization [9]. It was reported, that the acceleration of skeletal muscle growth is accompanied by a decrease in calpain activity, chiefly as a result of increased Calpastatin activity [10]. Some studies reported that significant correlations were noticed between the genetic variation within the sheep CAST gene and growth traits [3]; [11]; [12]; [13] and average daily weight gain [14]. So, the genetic difference of CAST gene between fat-tailed and thin tailed sheep breeds might be due to the effect of the CAST gene on fat deposition, displaying the linkage disequilibrium (LD) of this gene with quantitative trait loci (QTL) associated with fat deposition in chromosome 5 of sheep [1]. Some studies have been revealed the associations of the CAST polymorphism with carcass traits and meat quality in many livestock [15]; [16]; [17]; [18]; [19] and [19]. Recently, some of the SNPs in the CAST gene have been included as commercial genetic markers by some livestock industries [20]. It should be noted, that the existence of a similar association in sheep has been reported in only a few research papers to date [21]; [13]; [14]. Thus, the allegedly genetic variation in the CAST gene correlates with growth and potentially used as a candidate gene for growth and carcass traits in sheep. The purpose of this study is to investigate the CAST genotype and its association with growth traits in Batur sheep.

## 2. Materials and Methods

### 2.1. Animals and experimental design

Batur sheep are the predominant breed in the upland areas of Banjarnegara – Indonesia - where they are well adapted to the local cold and humid environment. This breed

developed by crossing between local breeds (Fat and Thin Tailed Sheep) and imported breed (Merino) [22]. Thirty heads of Batur sheep used for this experiment, the number of lambs born for every birth of each ewe was recorded, and the lambs' suckling program lasted for three months (90th day). Batur lambs were reared at the Batur area under an intensive feeding system until six months of age. All lambs were fed by concentrated feed consist of mixed feedstuff gave 3% of their body weight. Bodyweight measured monthly after weaning to six months of age.

## 2.2. DNA extraction

Blood samples (3 ml) were collected from each head of the experimental lambs' jugular vein and put into the vacutainer tubes containing EDTA (10 mL EDTA spray dried). For DNA extraction, 200 µl of whole blood samples were used and performed according to the manufacturer protocol (Genetika science). To amplify the 3rd exon region of the MSTN gene, then a specific primer is designed using the Primer3 software from the NCBI website in (Table 1). The concentration and purity of isolated DNA were measured using Nano-Drop 8000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) by Absorbance method.

TABLE 1: Primer forward and reverse for the CAST Amplification

| Amplified fragment | Size (bp) | Primer name | Primer sequence | Tm (oC) |
|---|---|---|---|---|
| CAST | 622 | Forward: | 5´-TGGGGCCCAATGACGCCATCGATG-3´ | Palmer et al. (1998) |
| | | Reverse: | 5´-GGTGGAGCAGCACTTCTGATCACC-3´ | |

## 2.3. Polymerase chain reaction conditions

Each 25 µl PCR reaction contained 25 ng of genomic DNA, 12.5 µl 2x Reaction mix of each primer, and 1.0 units of Taq DNA polymerase as shown in table 2, the cycling protocol was 5 minutes at 95°C as an initial denaturation, 35 cycles of denaturing at 94°C for 45 seconds, annealing at 73.9 for 45 seconds, extending at 72°C for 40 s, with final extension for 10 minutes. A PCR reaction mixture for amplification of the CAST gene was contained of Kapa mix 12.5 µL, Forward primer 1.0 µL, Reverse primer 1.0 µL, DNA template 1.0 µL, dH2O µL, with a total volume of 25 µL.

All samples were sent to Malaysia for sequencing, and the BioEdit program was used to identify a nucleotide substitution or mutation, and sequenced results compared with CAST gene reference (accession number AF016006.1) coding regions. The chromosome

ovr5 of the CAST region was used to identify a candidate gene by the Ensembl database (**www.ensembl.org**). A candidate gene was chosen based on their known function or potential involvement with growth and muscularity.

### 2.4. Data analysis

Genotypes and allele frequencies, heterozygosity rates were counted and a Chi-square test was performed. The mathematical model for gene and allele frequency [23] as follow:

**Genotype** frequency = $Xi = \frac{Gi}{N} \times 100\%$

Allele frequency = $Xij = \frac{2nii + nij}{2N}$

Where: Xi = Genotype or allele frequency, ith = homozygous alleles, jth = heterozygous alleles, Gi = number samples of i genotype, and N = total samples.

Statistical analysis using ANOVA procedure (software SPSS program version 17.0) was utilized to determine the correlation between CAST genotype and body weight and weaning weight.

## 3. Results and Discussion

### 3.1. CAST genotyping

The first step in studying the candidacy loci procedure is the genotyping of the base flock for the target gene. Batur sheep population revealed variation for the CAST gene; this assumed that the quality and quantity of meat in this breed could be affected by the CAST genotype, which leads us to study. The analysis revealed a total of sixteen polymorphic sites in the CAST coding region (Figure 1). There are four alleles observed (A, G, C, and T) every two alleles resulting in three genotypes, as in table 2. Sixteen polymorphic sites were observed in the fifth exon of the Cast regions, transversions at c.92T□G and c.285G>T loci, transitions at c.214G>A, c.280G>A, c.301C□T, and c.371A>G. one individual disrupted the reading frame in the whole CAST sequenced.

### 3.2. Population analysis in the CAST

In an investigated population, the population's genetic equilibrium was evaluated using χ2-test, and the CAST loci had χ2 value and probabilities presented in (table 3). Based on these results, the studied population showed a low degree of genotypic variability for

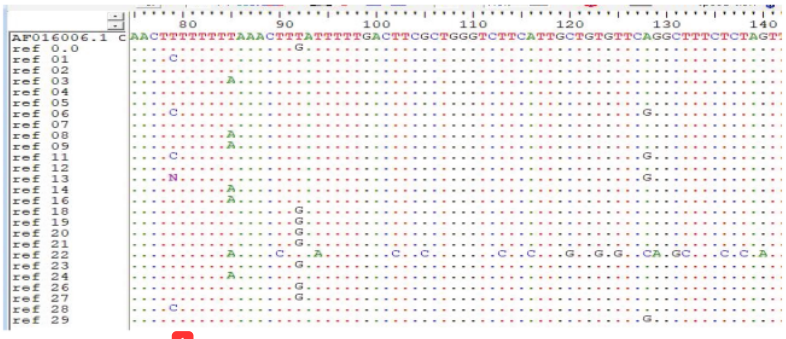

**Figure 1**: Chromatogram of BioEdit program used to identify if any nucleotide substitution in the CAST gene, all sequence plotted to a standard as a dot, compared to the reference sequence (NCBI accession number AF016006.1) there are 16 different variants on the matched sequence positions, one individual sequence disrupted reading frame in CAST gene..

the heterozygous CAST gene; thereby, the CAST gene of Batur sheep in Banjarnegara - Batur village was in Hardy-Weinberg equilibrium; thus, the null hypothesis is that the gametes unite at random.

A Large population in Hardy-Weinberg equilibrium is defined if the frequency of genotype (P2, 2pq, q2) and frequencies of allele (p and q) are constant from generation to generation because mating occurs roughly at random in a large population [24]; [25]. Selection is one factor that can alter the balance in the population rapidly [25]. Hardy-Weinberg equilibrium can be affected by inbreeding, assortative mating, natural selection, and population subdivision [23]. Calculated heterozygosity at the CAST loci in this sheep breed was low due to the close breeding system, which caused an increase in $\chi 2$ value.

TABLE 2: Number and genotype frequencies at exon 5 in the ovine CAST gene of Batur lambs.

| No | Nucleotide change | position | Transition/ transversion | Genotype | | |
|---|---|---|---|---|---|---|
| 1 | T⊠G | c.92 | Transversions | (18) TT | (08) TG | (00) GG |
| 2 | G>A | c.214 | Transitions | (17) GG | (09) GA | (01) AA |
| 3 | G>A | c.280 | Transitions | (17) GG | (09) GA | (00) AA |
| 4 | G>T | c.285 | Transversions | (16) GG | (10) GT | (00) TT |
| 5 | C⊠T | c.301 | Transitions | (18) CC | (08) CT | (01) TT |
| 6 | A>G | c.371 | Transitions | (15) AA | (10) AG | (01) GG |

In examined Batur lambs, the genotype frequency analysis showed the highest predominance of the "TT, GG, CC, and AA" genotypes with frequencies (66.7 %, 62.9 %, 59.3 %, 66.7 %, and 55.6 %) as homozygous while the heterozygous "TG, GA, GT, CT, and AG" genotypes were present at lower frequencies ((29.6 %, 33.3 %, 37 %, 29.6 %, and 37 %) respectively with four difference alleles (T, G, C, and A). It is known that the

using genetic marker depends on both the magnitude effect of a genotype frequency in the population. Several studies have reported the genetic variation in the bovine CAST gene, such as in coding regions [26], [27], and non-coding regions [10]; [28].

Table 3: Observed and expected genotypic and allele frequencies and χ2 test for the CAST loci

| Locus | Observed Genotype | | | Allele | | $X^2$ | p-value |
|---|---|---|---|---|---|---|---|
| c.92T>G | TT 0.667 | TG 0.296 | - | T 0.85 | G 0.15 | | |
| c.214G>A | GG 0.629 | GA 0.333 | AA 0.037 | G 0.80 | A 0.20 | | |
| c.280G>A | GG 0.629 | GA 0.333 | - | G 0.83 | A 0.17 | | |
| c.285G>T | GG 0.593 | GT 0.370 | - | G 0.81 | T 0.19 | | |
| c.301C>T | CC 0.667 | CT 0.296 | TT 0.037 | C 0.81 | T 0.19 | | |
| c.371A>G | AA 0.556 | AG 0.370 | GG 0.037 | A 0.77 | G 0.23 | | |
| | Expected Genotype | | | Expected Allele | | | |
| c.92T>G | TT 0.7 | TG 0.3 | GG 0.0 | T 0.85 | G 0.15 | 0.032 | 0.86 |
| c.214G>A | GG 0.6 | GA 0.3 | AA 0.0 | G 0.80 | A 0.20 | 0.0008 | 0.98 |
| c.280G>A | GG 0.7 | GA 0.3 | AA 0.0 | G 0.83 | A 0.17 | 0.042 | 0.84 |
| c.285G>T | GG 0.6 | GT 0.3 | TT 0.0 | G 0.81 | T 0.19 | 0.054 | 0.82 |
| c.301C>T | CC 0.7 | CT 0.3 | TT 0.0 | C 0.81 | T 0.19 | 0.0003 | 0.99 |
| c.371A>G | AA 0.6 | AG 0.3 | GG 0.1 | A 0.77 | G 0.23 | 0.007 | 0.94 |

According to [29] reported that in six (Thin-Tailed Sheep, Sukabumi, Jonggol, Kissar, Priangan, Margawati, and Fat-Tailed, Sumbawa, Donggala, and Rote) local sheep populations in Indonesia have CAST-1 and 2 alleles in the same frequency (48.4%) and spread throughout a population. Simultaneously, the CAST-3 was a rare allele and only found in the thin tailed sheep population (Sukabumi, Jonggol, and Kissar). Another study using PCR-RFLP reported the polymorphisms of Calpastatin (CAST-Msp1 locus) in local Indonesian sheep. With two types of alleles (M and N), but only found two types of genotypes (MN and NN) with the frequency of 25% and 75% for MN and NN genotypes, and 13% and 87% for M and N alleles [30]. Furthermore, this study has no exception.

## 3.3. Effect of CAST gene on growth traits

In the present study, exon 5 of the CAST gene is polymorphic. However, association analysis could be performed, and an attempt was made to verify the association of genotypic variants of the 5th exon with body weights at weaning and post-weaning gain (15, 30, 45, 60, 75, and 90 days) 6-months age,

the phenotypic and genotypic information. The descriptive statistic means ± standard error of weaning weight, repeated body weight, and average daily gain of different

TABLE 4: Means and Std. Error of body weight gain (ADG), weaning, and post-weaning of each genotype.

| Traits /SNPs | Body Weight (kg) | | | Average Daily Gain (g/d) | | |
|---|---|---|---|---|---|---|
| c.92T⊠G | TT | TG | GG | TT | TG | GG |
| Weaning | 19.9±1.0 | 20.9±1.7 | 00 | | | |
| 15-day | 23.9±1.4 | 24.9±2.1 | 00 | 69 | 99 | 00 |
| 45-day | 24.9±1.4 | 26.4±2.0 | 00 | 35 | 42 | 00 |
| 60-day | 25.5±1.5 | 27.0±2.2 | 00 | 186 | 220 | 00 |
| 75-day | 28.3±1.6 | 30.3±2.4 | 00 | 142 | 148 | 00 |
| 90-day | 30.4±1.6 | 30.4±2.4 | 00 | 45 | 119 | 00 |
| c.214G>A | GG | GA | AA | GG | GA | AA |
| Weaning | 20.5±.93 | 21.2±2.5 | 00 | | | |
| 15-day | 23.6±1.4 | 25.4±1.9 | 00 | 69 | 85 | 00 |
| 45-day | 24.7±1.4 | 26.7±1.9 | 00 | 30 | 51 | 00 |
| 60-day | 25.2±1.5 | 27.4±2.1 | 00 | 197 | 194 | 00 |
| 75-day | 28.1±1.7 | 30.3±2.3 | 00 | 139 | 155 | 00 |
| 90-day | 30.2±1.7 | 32.6±2.3 | 00 | 9 | -25 | 00 |
| c.280G>A | GG | GA | AA | GG | GA | AA |
| Weaning | 19.8±1.0 | 22.7±2.3 | 00 | | | |
| 15-day | 22.9±1.1 | 26.7±1.8 | 00 | 62 | 110 | 00 |
| 45-day | 23.8±1.1 | 28.4±1.8 | 00 | 23 | 64 | 00 |
| 60-day | 24.2±1.2 | 29.3±1.9 | 00 | 177 | 232 | 00 |
| 75-day | 26.8±1.3 | 32.8±2.1 | 00 | 153 | 127 | 00 |
| 90-day | 29.1±1.3 | 34.7±2.1 | 00 | 35 | -74 | 00 |
| c.285G>T | GG | GT | TT | GG | GT | TT |
| Weaning | 21.6±0.9 | 19.3±2.3 | 00 | | | |
| 15-day | 25.3±1.4 | 22.6±1.8 | 00 | 62 | 105 | 00 |
| 45-day | 26.2±1.4 | 24.2±1.8 | 00 | 25 | 56 | 00 |
| 60-day | 26.6±1.6 | 24.1±2.0 | 00 | 233 | 137 | 00 |
| 75-day | 30.1±1.7 | 27±2.1 | 00 | 150 | 134 | 00 |
| 90-day | 32.3±1.7 | 29±2.1 | 00 | -26 | 34 | 00 |
| c.301C⊠T | CC | CT | TT | CC | CT | TT |
| Weaning | 21.1±1.3 | 19.9±1.9 | 26.1±0.0 | | | |
| 15-day | 24.4±1.4 | 24.5±2.3 | 23.3±6.0 | 63 | 122 | -67 |
| 45-day | 25.3±1.4 | 26.1±2.3 | 23.2±6.1 | 33 | 60 | -74 |
| 60-day | 25.8±1.5 | 26.7±2.5 | 22.1±6.6 | 179 | 223 | 274 |
| 75-day | 28.5±1.7 | 30.2±2.7 | 26.2±7.2 | 146 | 170 | 67 |
| 90-day | 30.5±1.7 | 32.5±2.7 | 27.2±7.0 | 25 | -75 | 107 |
| c.371A>G | AA | AG | GG | AA | AG | GG |
| Weaning | 21.2±1.4 | 19.91.7 | 00 | | | |
| 15-day | 24.8±1.4 | 23.3±1.8 | 00 | 90.42 | 58 | 00 |
| 45-day | 26.2±1.4 | 24.1±1.8 | 00 | 33 | 44 | 00 |
| 60-day | 26.7±1.6 | 24.8±1.9 | 00 | 220 | 157 | 00 |
| 75-day | 29.9±1.7 | 27.2±2.2 | 00 | 135 | 158 | 00 |
| 90-day | 31.9±1.7 | 29.5±2.1 | 00 | 6 | -17 | 00 |

genotypes of Batur sheep in Banjarnegara at each CAST genotype loci were summarized in table 3. The presented results showed that the variations in the CAST gene at six different loci had no significant effect (p>0.05) on all of the studied growth traits (weaning weight, post-weaning body weight, post-weaning, average daily gain, chest girth, height at withers, and body length).

Inconsistent with our results, many studies explained significant effect for the variations in the Calpastatin gene on weaning weight and body gain in Eygytian Barki lambs [31], opposite to research reported by [32]; however, Post-weaning bodyweight till 6-month age of Batur sheep for the heterozygous genotypes at loci (c.92T□G, c.214G>A, c.280G>A, and c.301C□T ) slightly more massive than those carrying homozygous wild-type genotypes (c.285G>T and c.371A>G) with no significant differences (p > 0.5). Similarly, [14] reported an association of CAST genotype variations with body weight in Kurdi sheep, but did not affect growth properties when weaning until the age of six months. The Calpastatin gene's diversity has no association with body dimensions in Indonesian Thin Tailed Sheep but significantly affects the average daily gain ADG [33].

Changing the quality or quantity of feed offered can affect growth rate, management factors, and diseases as diarrhea has disadvantages reduced overall lamb's body weight, thereby production. In addition to that, changes in management and nutrition produce only temporary improvements required to be repeated each season. By contrast for genetic improvement of a trait contributing to lamb meat production is permanent.

TABLE 5: Significance of multivariate test of the CAST for all growth traits.

| Source | | Sum of square | d.f | Mean Square | F | Sig. | Partial Eta Squared |
|---|---|---|---|---|---|---|---|
| All growth traits vs genotype | Sphericity Assumed | 476.64 | 23 | 20.723 | 1.26 | 0.19 | 0.05 |
| | Greenhouse-Geisser | 476.64 | 2.85 | 167.099 | 1.26 | 0.29 | 0.05 |
| | Huynh-Feldt | 476.64 | 3.41 | 139.679 | 1.26 | 0.29 | 0.05 |
| | Lower-bound | 476.64 | 1.00 | 476.640 | 1.26 | 0.27 | 0.05 |
| Error of All growth traits | Sphericity Assumed | 9073.545 | 552 | 16.438 | | | |
| | Greenhouse-Geisser | 9073.545 | 68.5 | 132.540 | | | |
| | Huynh-Feldt | 9073.545 | 81.9 | 110.792 | | | |
| | Lower-bound | 9073.545 | 24.0 | 378.064 | | | |

TABLE 6: Significance of the multivariate test of the CAST for average daily gain.

| Source | | Sum of Squares | d.f | Mean square | F | Sig. | Partial Eta Squared |
|---|---|---|---|---|---|---|---|
| ADG vs genotype | Sphericity Assumed | 5.997 | 5 | 1.199 | 0.39 | 0.85 | 0.017 |
| | Greenhouse-Geisser | 5.997 | 2.24 | 2.674 | 0.39 | 0.70 | 0.017 |
| | Huynh-Feldt | 5.997 | 2.71 | 2.210 | 0.39 | 0.74 | 0.017 |
| | Lower-bound | 5.997 | 1.00 | 5.997 | 0.39 | 0.54 | 0.017 |
| Error of ADG | Sphericity Assumed | 353.15 | 115 | 3.071 | | | |
| | Greenhouse-Geisser | 353.15 | 51.6 | 6.845 | | | |
| | Huynh-Feldt | 353.15 | 62.4 | 5.659 | | | |
| | Lower-bound | 353.15 | 23.0 | 15.354 | | | |

ADG = average daily gain.

Bodyweight and growth traits are controlled by many genes (polygenic) and are greatly influenced by environmental factors [25]. The rate and degree of skeletal muscle growth are dependent on several factors, including the rate of muscle protein synthesis and degradation and the size and number of muscle cells. Calpain activities are required in the myoblast fusion process and the proliferation of cell growth [34]. Calpain system can also affect the number of fibers of the muscle cells in animals by affecting proliferation and modulating fusion myoblast. The system is also crucial in the normal growth of the skeletal muscles. The increase in the growth rate of skeletal muscle could be caused by a decrease in the rate of muscle protein degradation, and it is associated with a decrease in the calpain system's activity. This occurs due to a large increase in Calpastatin activity [34].

## 4. Conclusions

The present study investigated the CAST gene's allelic and genotypic effects on the growth traits in Batur sheep. Polymorphism is present in Batur sheep for the first time regarding assessing the association with the 5th exon of the CAST gene. The studied population was in Hardy-Weinberg equilibrium. There are four homozygous genotypes in the CAST "TT, GG, CC, and AA" with frequencies (66.7 %, 62.9 %, 59.3 %, 66.7 %, and 55.6 %) while the heterozygous "TG, GA, GT, CT, and AG" genotypes were shown the lower frequencies ((29.6 %, 33.3 %, 37 %, 29.6 %, and 37 %) respectively with four difference alleles (T, G, C, and A). Moreover, non-significance genotypic effects of the CAST gene in the studied growth traits. Present study supposed that the CAST gene's variation as selection tools to improve the growth traits in Batur sheep. However, the CAST gene might not be a useful marker for developing future

selection programs in Batur sheep unless further investigation of the CAST gene and its interactions performed with other genes involving muscle growth and carcass traits through analyzing association studies on a large scale.

## 5. Acknowledgement

This study has been supported by Jenderal Soedirman University, Program (Riset Institusi Unsoed No. P/495/UN23/14/PN/2019), Purwokerto, Indonesia.

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