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The Effect of SNP c.100800G > A on *CAST*|*Cfr*13I Gene Polymorphisms with Ultrasound Imaging of Meat Characteristics and Growth Traits in Bali Cattle

Research Article

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ABSTRACT

Bali cattle are known as native cattle from Indonesia, which commonly utilized as beef-producing animals. Calpastatin gene (*CAST*) plays essential role in meat quality. The aim of this study was to verify the effect of single-nucleotide polymorphism (SNP) c.100800G>A on the *CAST*|*Cfr*13I gene associated with meat characteristics and growth traits in Bali cattle. The meat characteristics, growth traits profile, and blood samples of Bali cattle (n=52 animals) obtained from BPTU Bali Cattle Denpasar, Bali Province. Comparison used were Belgian Blue (n=30 animals), Wagyu (n=7 animals), Limousin (n=14 animals), and Peranakan Onggole (PO) (n=30 animals). Ultrasound measurement was conducted to study the meat characteristics. The examination of the *CAST* gene polymorphisms used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method digested by *Cfr*13I enzyme and the analysis of effect of the CAST|*Cfr*131 gene on meat characteristics and growth traits in Bali cattle used t test with SAS 9.4 program. Bali cattle revealed polymorphic homozygous genotype (GG) and heterozygous genotype (GA) with allele frequencies of G and A were 0.923 and 0.077, respectively. In comparison, other breeds beef cattle, including Belgian Blue, Limousin, Wagyu, and PO, showed only the GG genotype with allele frequency of G was 1.000. The *CAST*|*Cfr*13I gene showed no significant association (P>0.05) with Bali cattle meat characteristics and growth traits. In conclusion, the SNP c.100800G > A may not be purposed as a marker for Bali cattle meat characteristics and growth traits. It was suggested that further study with a higher number of animals would be necessary to validate the effect of the SNP c.100800G > A on the *CAST* gene with the actual beef cutting of Bali cattle.

KEY WORDS Bali cattle, *CAST* gene, growth traits, meat characteristics, PCR-RFLP, ultrasound.

INTRODUCTION

Bali cattle is one of the Indonesian native cattle (Purwantara *et al.* 2012). Bali cattle has been registered by the Ministry of Agriculture of the Republic of Indonesia (325/Kpts/OT.140/1/2010) as a native Indonesian cattle. Analysis of the *MtDNA D-loop* gene also showed that Bali cattle have very close relatives with banteng, however, not with *Bos taurus* and *Bos indicus* (Wisesa *et al.* 2012). The use of Bali cattle as beef cattle still need to increase meat production and meat quality. This due to its adaptation to extreme condition (Purwantara *et al.* 2012). Therefore, Bali cattle is appropriate for environmental condition in Indonesia. Reproduction of Bali cattle is remarkable, in which the service/conception and birth rates of Bali cattle were 95.62% and 86.25%, respectively (Wawo, 2018). Suryanto *et al.* (2014) reported that the rib eye area (REA) of Bali cattle was 59.65 ± 0.64 cm^2, backfat thickness (BFT) was 3.08 ± 0.28 mm, and the marbling score (MS) was 2.65 ± 0.92%. Setiaji *et al.* (2019) also described that the birth

weight (BW) of Bali cattle was 17.94 ± 1.95 kg, weaning weight (WW) 82.52 ± 15.24 kg, and yearling weight (YW) 122.46 ± 18.29 kg. Many factors affect meat quality, i.e. gender, age, feed, breeds, housing system, pre-slaughter treatment, and genetics (Raza *et al.* 2019). Gen *CAPN*, *CAST*, *COL1A1*, and *ASAP1* were reported to associate with meat quality in Nellore cattle using the GWAS Illumina BovineHD Beadchip technique (Tizioto *et al.* 2013). Calpastatin (*CAST*) is one of the genes that has essential role in meat quality in swine (Krzęcio *et al.* 2008); poultry (Ghamari Monavvar *et al.* 2020); cattle (Jr *et al.* 2018; Raza *et al.* 2019). The *CAST* was identified in muscle tissue as a μ-calpain inhibitor (Busch *et al.* 1972) and had an important role in the meat tenderization process (Pinto *et al.* 2010). The *CAST* gene is located on chromosome 7 in cattle (Kappes *et al.* 1997), which consists of 35 exons and 34 introns (Raynaud *et al.* 2005). The *CAST* gene also contributes in the postmortem proteolysis (Calvo *et al.* 2014). The higher *CAST* activity (before and after rigor mortis) lowering the number of myofibrillar protein postmortem proteolysis (Guimarães *et al.* 2019), which causes the meat to become tougher. The *CAST* also influences in gene expression during cell development (Van Ba *et al.* 2014). Furthermore, the calpain/calpastatin system regulates muscle cell migration and cell differentiation in the early stages of development (Dedieu *et al.* 2004; Moyen *et al.* 2004; Barnoy *et al.* 2005). Several reports showed that the *CAST* gene is also associated with birth weight (BW) and weaning weight (WW) in Argentine Angus cattle (Pintos and Corva, 2011).

Studies regarding to the polymorphisms of *the CAST* gene still need to be carried out in confirming the association between the *CAST* gene and meat characteristics and growth traits. Some of the methods commonly used in DNA level studies are Restriction Fragment Length Polymorphisms (RFLPs), Amplified Fragment Length Polymorphisms (AFLPs), Random Amplified Polymorphic DNA (RAPD), Real-time PCR, Sequencing, and DNA microarrays (Pereira *et al.* 2008). The PCR-RFLP is a method used to examine genetic diversity between species using restriction enzymes (Sun and Lin, 2003). The PCR-RFLP method is efficient, especially for specific loci (Hashim and Al-Shuhaib, 2019). Pratiwi (2016) reported 25 SNPs on the *CAST* gene, which were polymorphic in Bali cattle using the direct sequencing method. Among the SNPs is c.100800G > A significantly affected the rump thickness with a limited population. There is still lack study of the SNP c.100800G > A on several beef cattle breeds. Based on these rationales, this study was conducted to identify and analyze the diversity of SNP c.100800G > A on the *CAST* gene using PCR-RFLP measure on several beef cattle breeds and its association with meat characteristics and growth traits.

MATERIALS AND METHODS

Animal and traits evaluated

Blood samples from 133 several breeds of beef cattle were used in this study. T including Bali cattle (n=52) blood samples were obtained from BPTU-HPT Bali Cattle Denpasar, Bali Province, Belgian Blue (n=30), Wagyu (n=7), Peranakan Onggole (PO) (n=30), Limousin (n=14) blood samples were used as comparisons. About 300 μL of the blood samples were extracted by standard method from Geneaid™ DNA Isolation Kit Ver. 02.21.17. The meat characteristic traits in Bali cattle, including backfat thickness (BFT), longissimus dorsi thickness (LDT), percentage of intramuscular fat/IMF (PIMF), and marbling score (MS) were measured by ultrasound imaging method (Veterinary Ultrasound Scanner WED-3000V models) at the 12^{th}-13^{th} ribs position (Melendez and Marchello, 2014). The USG results were examined using Image-J NIH software (ImageJ®, NIH, USA). To determine the MS is based on AUSTRALIAN MEAT and MSA (http://www.wagyu.org.au/marbling/). The growth traits in Bali cattle were figured by birth weight (BW), weaning weight (WW), yearling weight (YW), two years weight (TYW), and daily gain weight (DGW).

PCR Amplification and gene polymorphism

The fragment *CAST* gene was amplified using specifics primer (Palmer *et al.* 1998; Putri *et al.* 2015) (GenBank: AF117813.1) exon 15 and 16, forward: 5'TGGGGCCCAATGACGCCATCGATG'3 and reverse: 5'GGTGGAGCAGCACTTCTGA TCACC'3. The amplification of *CAST* gene fragment was performed in PCR Thermal Cycler-ESCO with the following protocol: the predenaturation temperature at 95 ˚C for 1 min, followed by 35 cycles of denaturation at 95 ˚C for15 s, annealing at 60 ˚C for15 s, extension at 72 ˚C for 10 s, and the final extension was at 72 ˚C 5 min. Amplification was examined on horizontal electrophoresis (100 volts for 40 min) in 1.5% agarose gel and visualized by UV transilluminator (Biorad™, California, USA). The 624 bp *CAST* gene was digested by *Cfr*13I restriction enzyme, which has 5'-G|GNCC-'3 digestion site (https://nc2.neb.com/NEBcutter2/). The reaction mixture was incubated at 37 ˚C for 4 h. The digestion products were examined by horizontal electrophoresis (100 volts for 40 min) in 2% agarose gel and visualized by UV transilluminator (Biorad™, California, USA).

Data analysis

The allelic (X_i) and genotypic (X_{ii}) were calculated according to Moonesinghe *et al.* (2010) by using Pop-gen 1.32 program. The mathematical model as follow:

$X_i = (N_{ii} + Sn_{ij}) / 2N$
$X_{ii} = n_{ii} / n$

Where:
X_i: allelic frequency of G and A.
$X_i i$: genotype frequency GG and GA.
n_{ii}: number of individual of GG genotype.
n_{ij}: number of individual of GA genotype.
N: total of individual samples.

The observed heterozygosity (H_o) and expected heterozygosity (H_e) calculated using the method of Moonesinghe *et al.* (2010) using the PopGen 1.32 program. The mathematical model as follow:

$$H_o = \sum_{i=j}^{N} \frac{n_{ij}}{N}$$

$$H_e = 1 - \sum_{i=1}^{q} X_i^2$$

Where:
H_o: observed heterozygosity.
H_e: expected heterozygosity.
n_{ij}: number of heterozygous individuals.
N: total sample.
X_i: frequency of the *i* allele.
Q: number of the allele.

Chi-Square was used to test the Hardy-Weinberg balance (Moonesinghe *et al.* 2010) by using Pop-gen 1.32 program. The mathematical formula as follows:

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

Where:
X^2: chi-square.
O: observed value.
E: expected value.

The association between *CAST*|*Cfr*13I gene with meat characteristics (backfat thickness/BFT, longissimus dorsi thickness/LDT, percentage of *intramuscular fat*/PIMF, and marbling score/MS) and growth traits (birth weight/BW, weaning weight/WW, yearling weight/YW, two years weight/TYW and daily gain weight/DGW) were analyzed by using t test (Kim, 2015) with SAS 9.4. program (SAS, 2004). The mathematical model as follow:

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{s\sqrt{\left(\frac{1}{n_1}\right) + \left(\frac{1}{n_2}\right)}}$$

Where:

$$s = \sqrt{\frac{\sum_{i=1}^{n}(\bar{x}_i - \bar{x}_1)^2 + \sum_{i=1}^{n}(\bar{x}_i - \bar{x}_2)^2}{n_1 + n_2 - 2}}$$

Where:
$\bar{X}_1$: mean meat characteristics/growth traits of the GG genotype.
$\bar{X}_2$: mean meat characteristics/growth traits of the GA genotype.
n_1: number of individual with GG genotype.
n_2: number of individual with GA genotype.
s: variety.

RESULTS AND DISCUSSION

The *CAST* gene was successfully amplified on exon 15 to exon 16 with fragment size 624 bp on annealing temperature of 60 ˚C (Palmer *et al.* 1998); (Pratiwi, 2016) for 15 s (Figure 1). The success of the DNA amplification process is influenced by primer concentration, denaturation duration, amplification temperature, DNA samples, and $MgCl_2$ concentration (Williams, 2005). PCR-RFLP product of *CAST* gene digestion by the *Cfr*13I enzyme produced GG genotype and the GA genotype in Bali cattle (Figure 2). GG genotype was homozygous genotype with fragments size of 282 bp and 342 bp, while genotype GA was heterozygous genotype with fragments size of 282 bp, 342 bp, and 624 bp. The digestion site of the *Cfr*13I enzyme was 5'-G|GNCC-3' shown in Figure 3. In comparison, GA genotype was not found in Belgian Blue, Wagyu, PO, and Limousin. The SNP c.100800G > A on the *CAST* gene was specific only found in Bali cattle. Li *et al.* (2010) reported that the GG genotype of the *CAST* gene was not found in Qinchuan cattle (*Bos taurus*). Braunvieh three-way cross (*Bos indicus*×*Bos taurus*) cattle in 5th intron of the *CAST* gene produced two genotypes, there were CC genotype and CG genotype, while the GG genotype was not found in this cattle (Curi *et al.* 2010). The results of this study were the same as reported by Pratiwi (2016) that GG and GA genotypes were obtained because of transition mutation of the purine base guanine (G) with the purine base adenine (A). The mutation occurred at position 282 bp of the *CAST* gene fragment in Bali cattle. However, Putri *et al.* (2015) reported that the genotypes *CAST* gene GG and AG were found in Bali cattle.

The GG genotype frequency was 0.850, higher than the GA genotype was 0.150, while the G and A allele frequencies were 0.923 and 0.077, respectively.

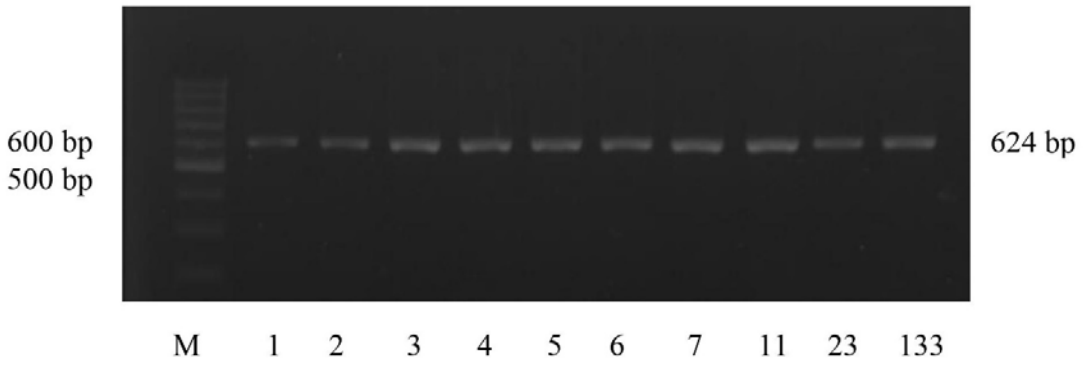

Figure 1 The result of Calpastatin gene PCR fragments in 1.5% agarose gel electrophoresis
Note: M (marker), 1-133 (number of animals)

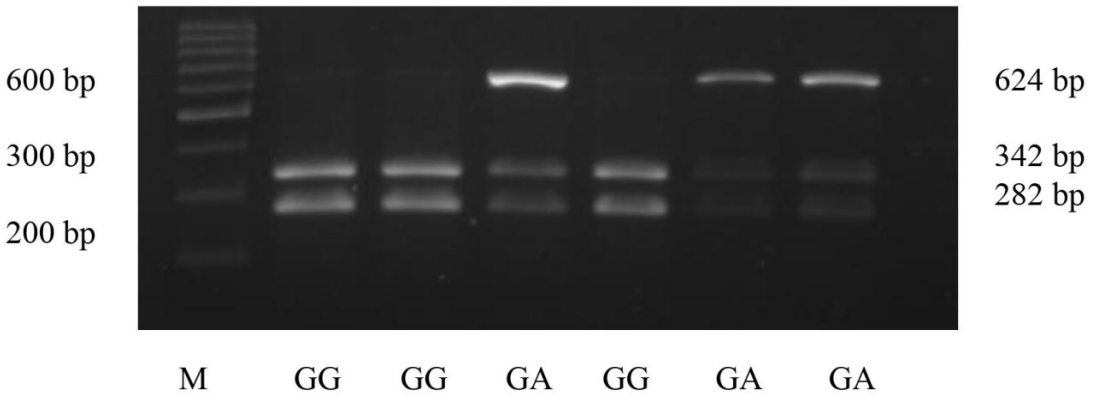

Figure 2 The result of *CAST/Cfr*13I fragment restriction in 2% agarose gel electrophoresis
Note: M (marker), GG and GA (genotypes)

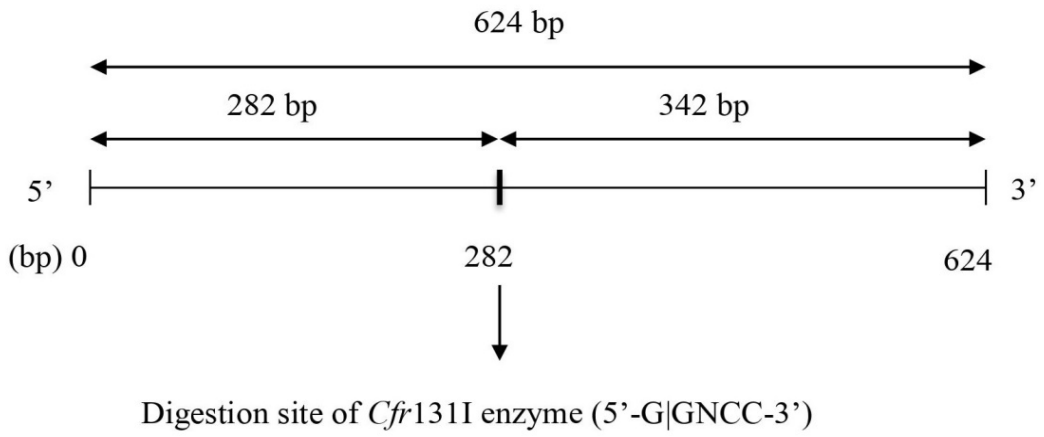

Figure 3 Digestion site of the *Cfr*13I enzyme on the *CAST* gene

The G allele frequency was higher than the A allele frequency because the AA genotype was not found in the Bali cattle population in this study. According to Putri *et al.* (2015) an allele polymorphic has a value ≥ 0.01, which indicated that Bali cattle was polymorphic, while Belgian Blue, Wagyu, PO, and Limousin were monomorphic (Table 1).

The genetic diversity in the population based on allelic frequency measured by heterozygosity (Ismail *et al.* 2020). The observed heterozygosity (H_o) was 0.154 and the expected heterozygosity (H_e) was 0.142. Volkandari *et al.* (2017) reported that if H_o higher H_e means the population was diverse, this indicates that genetics in Bali cattle was diverse. Noor (2010) reported that genetic diversity could be used in the breeding program, where selection is necessary for a diverse population and crossing is necessary for a uniform population. Furthermore, if the $H_o > H_e$ indicated random mating n the population, whereas if $H_o < H_e$ indicated inbreeding (Chesnokov and Artemyeva, 2015). Therefore, random mating occurred in Bali cattle population, while other breeds of beef cattle could not be calculated because the allele was monomorphic.

The population was in Hardy-Weinberg equilibrium (HWE) or not analyzed by chi-square test (χ^2). The population was stated to be balanced if the calculated χ^2 value was smaller than χ^2 table ($P<0.05$) (Allendorf *et al.* 2010). Bali cattle in this study were in the HWE, while breeds of beef cattle could not be calculated because the allele was monomorphic. The study indicated that the genetic frequency in Bali cattle in this study was in a balanced condition and the genetic frequency was relatively stable. Allendorf *et al.* (2010) reported that the population was in HWE because the genotypic and allelic frequency does not change from generation to generation.

The results of ultrasound imaging of meat characteristics parameters (BFT, LDT, MS, and PIMF) are represented in Figure 4. The ultrasound can be carried out to determine carcass composition since the method was simple, viable, effective, fast, and accurate without sacrificing the animal (Lambe *et al.* 2010). Melendez and Marchello, (2014) reported that ultrasound could be utilized to presume carcass in cattle with 70-85% accuracy. Similarly, Jakaria *et al.* (2017) reported that the ultrasound could be applied to determine characteristics of carcass quality in Bali cattle with a high correlation coefficient ranged between 0.291-0.938.

Association analysis was only performed on Bali cattle because Bali cattle were polymorphic, while Belgian Blue, Wagyu, PO, and Limousin cattle were not calculated due to the alleles were monomorphic. The mean value and standard deviation of each meat characteristics (BFT, LDT, MS, and PIMF) were shown in Table 2.

Based on the Table 2, the LDT for GA genotype (53.03±6.10 mm) higher than GG genotype (51.99±5.03 mm). The BFT for GA genotype (2.38±1.03 mm) higher than GG genotype (2.19±0.82 mm). According to Putri *et al.* (2015) BFT range from 1 up to 5 mm classified as an ideal carcass for the traditional market in Indonesia. Moreover, for the MS and PIMF GA genotype were 1.24 ± 1.03 and 1.94 ± 1.03%, respectively better than the MS and PIMF of for the GG genotype were 1.17 ± 0.31 and 1.72 ± 1.03%, respectively. BFT in this study was higher, but MS and PIMF were lower than what have been reported by Pratiwi (2016) in the same SNP. This was because marbling is the last adipose tissue to be deposited in animals, although adipose tissue begins to be deposited during the weaning period (Hocquette *et al.* 2010).

The results showed that the polymorphisms of SNP c.100800G > A with GG genotype and GA genotype in Bali cattle showed no significant association ($P>0.05$) with meat characteristics (LDT, BFT, MS, and PIMF). This might be the SNP is located in the intron region (non-coding RNAs). The non-coding RNA transcribed from the intron region was involved in different biological processes, including transcription control and post-transcription of gene expression affect the phenotypic alteration (Nakaya *et al.* 2007). The meat characteristics of GA genotype, which had average LDT, BFT, MS, and PIMF higher than GG genotype, despite not statistically significant. Moreover, calpain/calpastatin activity occurs during the postmortem, which plays a role in the myofibrillar protein degradation, and fewer sarcoplasmic proteome changes during aging (Guimarães *et al.* 2019). On the same SNP Pratiwi (2016) reported that the *CAST* gene associated rump thickness.

Several studies had stated about the *CAST* gene in different SNP and different cattle. Li *et al.* (2013) described that the *CAST* gene had potential as a candidate gene for cooking loss and meat color in Yanbian cattle (*Bos taurus*). In Hanwoo cattle (*Bos taurus*), *CAST*: c.1985G > C had a significant impact on tenderness by using the Warner-Blatzler Shear Force (WBSF) method (Lee *et al.* 2014). Research by Li *et al.* (2010) explained that in Chinese Commercial Cattle, the *CAST* gene had no significant effect on slaughter weight, carcass weight, rib eye area (REA), raw shrinkage weight, MS, and BFT, but significantly influenced meat tenderness. In accordance with Curi *et al.* (2010) stated that the *CAST*|*Rsa*I gene had no significant effect the REA, BFT, PIMF, and myofibrillar fragmentation index (MFI) in Chinese Commercial Cattle. Another study conducted by Enriquez-Valencia *et al.* (2017) reported that the *CAST* gene had no significant effect on REA, BFT, and total fat in Nellore cattle (*Bos indicus*). Putri *et al.* (2015) reported different results that *CAST* gene associated with LDT and BFT in Bali cattle.

Table 1 Genotypic frequencies, allelic frequencies, observed heterozygosity (H_o), expected heterozygosity (H_e) and Hardy-Weinberg equilibrium (χ^2)

Breeds	Genotype frequency		Allelic frequency		H_o	H_e	χ^2
	GG	GA	G	A			
Bali	0.850	0.150	0.923	0.077	0.154	0.142	0.310^{ns}
Belgian blue	1.000	0.000	1.000	0.000	0.000	0.000	0.000^{nd}
Wagyu	1.000	0.000	1.000	0.000	0.000	0.000	0.000^{nd}
Peranakan Onggole (PO)	1.000	0.000	1.000	0.000	0.000	0.000	0.000^{nd}

NS: non significant α 0.05 (χ^2 <3.84) and ND: not counted because the alleles were monomorphic.

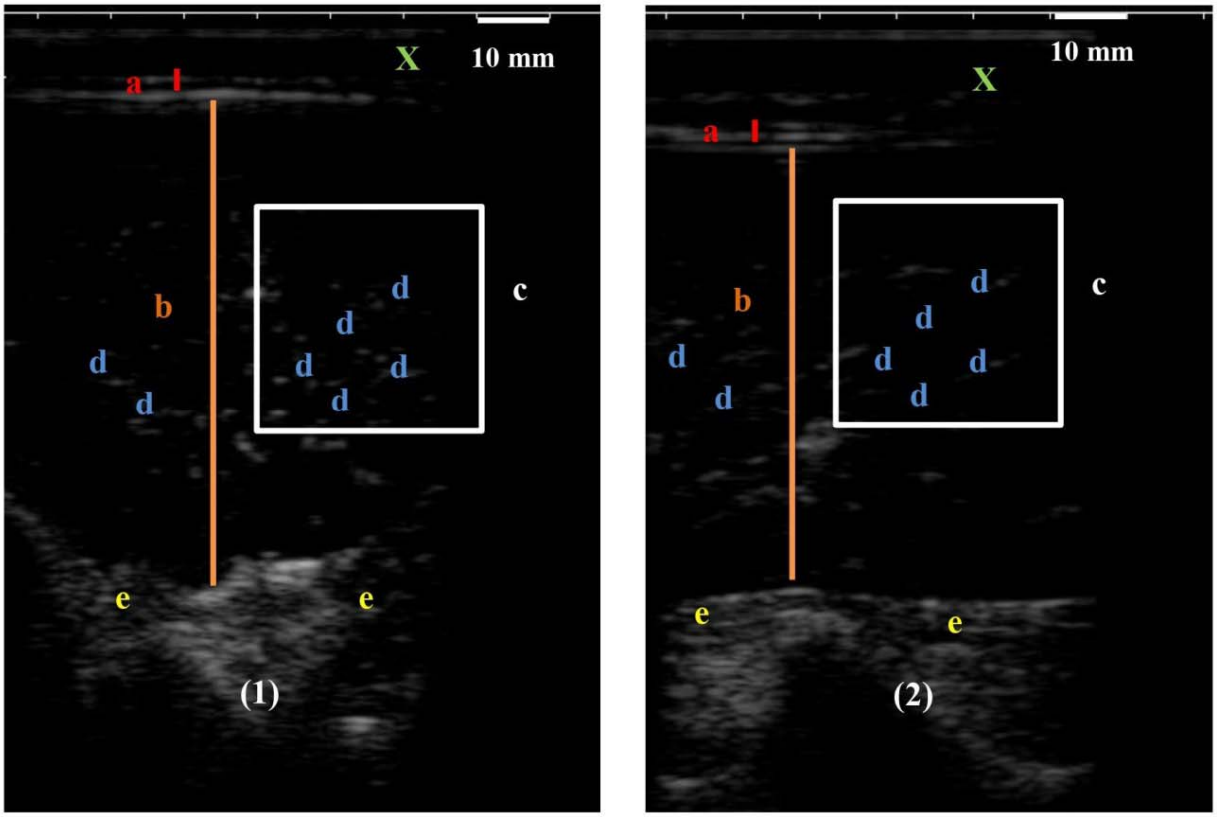

Figure 4 Ultrasound of the 12-13th ribs in (1) vertical/transversal and (2) horizontal/longitudinal positions
(a) back fat thickness; (b) longissimus dorsi thickness; (c) percentage of IMF measurement area 15 × 15 mm; (d) IMF; (e) bone and (X) dermis

This might be due to the mutation site used in Putri *et al.* (2015) was different from that used in this study.

Growth traits were featured by birth weight (BW), weaning weight (WW), yearling weight (YW), two years weight (TYW), and daily gain weight (DGW). The mean value and standard deviation of each growth traits were shown in Table 3.

Based on the Table 3, the GA genotype has higher BW, WW, YW, TYW, and ADG than the GG genotype. This was due to the lower decomposition of fat during the growth period, and expand during the fattening period therefore the concentration of fat in muscle (IMF content) will rise (Hocquette *et al.* 2010).

In addition, the increase in *CAST* activity may inhibit muscular fiber degradation, thereby resulting in the further accumulation of muscle mass (MacHado *et al.* 2020).

The association of the SNP c.100800G > A *CAST* gene with growth traits included BW, WW, YW, TYW and DGW showed that GG genotype and GA genotype in Bali cattle showed no significant association (P>0.05) with growth traits. We assumed that the detection of polymorphisms was in the intron region (15th intron) that causing silent mutations (Nikmard *et al.* 2012). We selected the partial sequence from 15th up to16th exons, while it seems these partial sequences have not been the appropriate parts for observation of polymorphism in Bali cattle.

Table 2 Association of *CAST* gene with with ultrasound imaging of meat characteristics Bali cattle

Meat characteristics parameter	Genotype		P-value
	GG (n=44)	GA (n=8)	
LDT (mm)	51.99±5.03	53.03±6.10	0.662^{ns}
BFT (mm)	2.19±0.82	2.38±1.03	0.626^{ns}
MS	1.17±0.31	1.24±0.23	0.469^{ns}
PIMF (%)	1.72±0.73	1.91±0.55	0.416^{ns}

LDT: longissimus dorsi thickness; BFT: backfat thickness; MS: marbling score and PIMF: percentage of intramuscular fat/IMF.
NS: non significant.

Table 3 Association of *CAST* gene with growth traits in Bali cattle

Growth traits parameter[1]	Genotype		P-value
	GG (n=44)	GA (n=8)	
BW (kg)	18.41±5.41	18.50±2.98	0.946^{ns}
WW (kg)	83.60±28.40	88.30±25.60	0.650^{ns}
YW (kg)	179.00±58.80	188.1±50.50	0.658^{ns}
TYW (kg)	246.40±67.10	285.40±69.40	0.176^{ns}
ADG (kg/day)	0.307±0.07	0.308±60.07	0.176^{ns}

BW: birth weight; WW: weaning weight; YW: yearling weight; TYW: two years weight and ADG: average daily gain.
NS: non significant.

Several studies in different animals reported by Gorlov *et al.* (2016) explained that the *CAST*|*Msp*I gene was associated with BW, WW, and DGW in Salsk sheep. Whereas, Putri *et al.* (2015) described that the *CAST*|*Alu*I gene was not associated with BW, shoulder height, chest circumference, body length, and ADG in Bali cattle. Calpains/calpastatin system plays a role in muscle cell migration and differentiation in the early stages of development (Dedieu *et al.* 2004; Moyen *et al.* 2004; Barnoy *et al.* 2005). Nevertheless, Pintos and Corva (2011) identified that the *CAST* gene had significant effect BW and WW in Argentinian Angus cattle. The absence of an association may be due to the small sample size of Bali cattle used in this study compared to the research of Pintos and Corva (2011) which used 1.365 Black and Red Angus bulls.

CONCLUSION

There was no polymorphism SNP c.100800G > A on the *CAST* gene observed in Belgian Blue, Wagyu, PO, and Limousin. The association study of SNP c.100800G > A on the *CAST* gene had no significant impact on the meat characteristics and growth traits using ultrasound. The SNP may not be purposed for a selection tool in Bali cattle. Therefore, the future study with a higher number of animals will be necessary to validate the effect of the SNP c.100800G > A on the *CAST* gene with the actual beef cutting of Bali cattle.

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