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The favorable alleles of *AKIRIN2:c.*188G>A, EDG1:c.-312A>G* and *TTN:g.231054C>T* as candidate markers for high-marbling are very low in Bali cattle

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Abstract. *Anwar S, Wulandari AS, Putra WPB, Said S. 2019. The favorable alleles of AKIRIN2:c.*188G>A, EDG1:c.-312A>G and TTN:g.231054C>T as candidate markers for high-marbling are very low in Bali cattle. Biodiversitas 20: 965-970.* Marbling is one of primary factors influencing the palatability of beef. The *AKIRIN2*, *EDG1* and *TTN* genes are considered as the three candidate genes responsible for high-marbling in beef cattle. This study aimed to investigate the genetic polymorphism of the SNPs of *AKIRIN2:c.*188G>A, EDG1:c.-312A>G* and *TTN g.231054C>T* in Bali cattle. A total of 125 DNA samples of Bali cattle were genotyped for *AKIRIN2* and *TTN* gene and 82 DNA samples for *EDG1* gene using PCR-RFLP method. In the present study, it was found that the *A* allele of *AKIRIN2:c.*188G>A*, the *G* allele of *EDG1:c.-312A>G* and the *T* allele of *TTN g.231054C>T* that considered as the favorable alleles were found to be very low in Bali cattle (0.004, 0.000, and 0.004, respectively). This results indicated that the polymorphism of *AKIRIN2:c.*188G>A, EDG1:c.-312A>G* and *TTN g.231054C>T* were very low and can be considered as monomorphic in Bali cattle. These findings suggested that Bali cattle may not genetically potential as a high-marbling cattle breed. However, the other specific genes for high-marbling still needs to be investigated in Bali cattle.

Keywords: *AKIRIN2*, Bali cattle, *EDG1*, high-marbling, *TTN*

INTRODUCTION

Bali cattle (*Bos javanicus*) as native cattle of Indonesia is known to have various advantages such as able to adapt to harsh environments, able to utilize low-quality feed efficiently and have a higher level of fertility and conception rate compared to another cattle breeds (Martojo 2003; Purwantara et al. 2012). However, based on the aspect of eating quality of meat, Bali cattle's meat is less qualified because it is considered less tender than those from *Bos taurus* and *Bos taurus* crossbred cattle (Suwiti et al. 2015; Khasrad et al. 2017). Tenderness is recognized as one of the major important attributes for eating quality besides the juiciness and flavor (Okumura et al. 2007; Corbin et al. 2015). As for this tenderness, the less-tender Bali cattle's meat may be caused by the absent of or low level of marbling (Oematan 2000; Sri Rachma and Harada, 2010; Suryanto et al. 2014; Pratiwi et al. 2016). Nevertheless, more tender meat can be obtained by increasing the marbling level (Platter et al. 2003; Corbin et al. 2015). It will be useful to obtain juicier and tender meats of Bali cattle.

Variation of marbling level in beef cattle is majority affected by the genetic factor as indicated on moderate to high heritability and significantly has different level between breeds (Harper and Pethick 2001; Johnston and Graser 2010; Yamada 2014). This suggested that genetic selection is an effective way to control the variation of marbling traits in cattle. However, conventional selection methods for the traits that difficult to measure such as marbling level, considered being less effective. Molecular genetics technology through the marker-assisted selection (MAS) could be used to assist in enhancing the accuracy of conventional selection which is giving more effectiveness and efficiency in selection (Dekkers 2004).

Exploration of specific genes that responsible for high-marbling is expected to be used for efficiency in MAS program to improve marbling level in Bali cattle. However, in a previous study, Anwar et al. (2017) did not find the favorable *T* allele at 5'-untranslated region (5'UTR) of *thyroglobulin* gene (*422T>C* SNP) in pure breeding location of Bali cattle. The *AKIRIN2*, endothelial differentiation sphingolipid G-protein-coupled receptor 1 (*EDG1*) and TITIN (*TTN*) genes are the three of the most promising positional functional candidate gene that can be used to achieve these purposes since they were detected to have different expression patterns in *musculus longissimus* muscle between low- and high-marbled steers groups (Sasaki et al. 2006; Sasaki et al. 2009; Yamada et al. 2009a). Furthermore, *AKIRIN2* and *TTN* genes were regarded to have a direct impact on marbling (Watanabe et al. 2011).

In bovine, the *AKIRIN2*, *EDG1* and *TTN* genes have been mapped in Japanese Black cattle to chromosome 9, 3 and 2, respectively (Yamada et al. 2006). Based on SNPs identification and association studies, the SNPs of *c.*188G>A* at 3'-untranslated region (3'UTR) of *AKIRIN2* (*AKIRIN2:c.*188G>A*), *c.-312A>G* at 5'-untranslated region (5'UTR) of *EDG1* (*EDG1:c.-312A>G*) and *g.231054C>T* at the promoter region of *TTN* gene

(*TTN:g.231054C>T*) have been found significantly associated to high-marbling breeding value (Sasaki et al. 2009; Yamada et al. 2009a, b; Sukegawa et al. 2014).

The information of genetic polymorphism of the genes responsible for high-marbling is important to identify to find out the genetic potential and evaluation in Bali cattle breeding program related to marbling traits. The study of *AKIRIN2*, *ADG1*, and *TTN* gene polymorphisms as three candidate genes for high-marbling have not been investigated in Bali cattle. Therefore, the aim of this study is to investigate the genetic polymorphism of the SNPs of *AKIRIN2:c.*188G>A*, *EDG1:c.-312A>G* and *TTN g.231054C>T* in Bali cattle.

MATERIALS AND METHODS

Animals and DNA Samples

A total of 125 genomic DNA samples of Bali cattle were used and prepared as a part of samples of the previous study (Anwar et al. 2016; Anwar et al. 2017). The animals were mostly raised under extensive management system that grazed freely in grasslands, plantations or post-harvested paddy fields. All 125 individuals were used to detect *AKIRIN2:c.*188G>A* and *TTN:g.231054C>T*, while 82 of 125 samples were used to detect *EDG1:c.-312A>G*.

DNA Amplification

In the present study, the position of targeted SNPs from three candidate genes was confirmed based on the latest bovine genomic reference sequences (RefSeq). The primer used was based on Sasaki et al. (2009), Yamada et al. (2009b) and Yamada et al. (2009a) to amplify the targeted fragments of *AKIRIN2*, *EDG1* and *TTN* genes, respectively. The detailed information of SNPs position, genomic reference sequences and pair of primers used in the present study are shown in Table 1.

DNA amplification was performed by polymerase chain reaction (PCR) method in a final volume of 10 µL containing approximately 10-12 ng genomic DNA template, 5 µL of MyTaq^{TM} HS Red Mix, 2x (Bioline, USA), 2 µM of each primer and ddH_2O up to 10 µL of final volume. Amplification cycling conditions involved initial denaturation at 95°C for 1 min, followed by 35 cycles denaturation at 95°C for 15 s, annealing (depends on each gene) for 15 s, extension at 72°C for 10 s and a final extension at 72 °C for 5 min. The annealing temperature were 53, 65.5 and 52°C for *AKIRIN2*, *EDG1*, and *TTN* genes, respectively. The PCR products were separated by electrophoresis in 1.5% agarose gel and stained with GelRed® 10,000X in water (Biotium, USA) and then visualized under a G-BOX Gel Documentation System (Syngene, UK) to confirm the presence of PCR products.

Genotyping by PCR-RFLP

Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The PCR products containing targeted SNPs were digested using restriction enzyme *FokI*, *Msc*I and *Hpy*CH4III (New England Biolabs, USA) to detect polymorphism of *AKIRIN2:c.*188G>A*, *EDG1:c.-312A>G* and *TTN:g.231054C>T*, respectively. The digested products were separated by electrophoresis in 3% agarose gel and stained with GelRed®10,000X in water (Biotium, USA) and then visualized under a G-BOX Gel Documentation System (Syngene, UK) to determine genotypes.

Table 1. Information of SNPs position, genomic reference sequences and pair of primers used to amplify the targeted fragments of *AKIRIN2*, *EDG1* and *TTN* genes in Bali cattle

Gene	SNP	Primer sequences (5'to 3')[a]	Genomic reference sequences				Fragment size (bp)
			Previous study[b]		Present study[c]		
			Acc. no.	SNP position	Acc. no.	SNP position[d]	
AKIRIN2	*G>A*	F : TCTTAGGCAGCAACCGGATT R: GAAGGGCATGTTCTTAGAATACCAG	NW_001495576	*c.*188*	NC_037336.1	g.62261408	170
EDG 1	*A>G*	F: GTCTCAGCTGCACAGATCC R: GAAGACCTCCGGCCGCGAT	NC_007301.5	*c.-312*	NC_037330.1	g.42039159	378
TTN	*C>T*	F: TCATCTCCTAACTACTTCCCA R : ACAAAATCTGAACCTGGCTT	NW_001494580	*g.231054*	NC_037329.1	g.18060564	227

Note: [a] Pair of primers (forward and reverse) for *AKIRIN2*, *EDG1* and *TTN* genes designed by Sasaki et al. (2009), Yamada et al. (2009b) and Yamada et al. (2009a), respectively; [b] Bovine genome reference sequences used in previous study: NW_001495576 (Sasaki et al. 2009), NC_007301.5 (Shin and Chung 2012), NW_001494580 (Yamada et al. 2009a); [c] Bovine genome reference sequences used in the present study; [d] These SNP positions are relative to the SNP positions in previous study.

DNA sequencing analysis

In order to confirm the polymorphic sites of both in *AKIRIN2*, *EDG1* and *TTN* genes, PCR products representing each genotype were sequenced in both directions using ABI PRISM 96-capillary 3730xl DNA Analyzer (Applied Biosystem, USA). For the sequencing of *AKIRIN2* gene, a new pairs of primer was designed based on Refseq accession NC_037336.1 to amplify a 458 bp fragment containing SNP of *c.*188G>A* (forward: 5'-TTGTTCCTTGTTGAGTTGTTGC -3' and reverse : 5'-ATACAGAAATGCCCTCCTCCTC -3'), because the fine sequence resulted from the fragment with designed primers by Sasaki et al. (2009) is too short and does not include the targeted SNP. Polymerase chain reaction was performed with the annealing temperature at 63°C. The sequencing result was then aligned with the reference sequences and analyzed using Bioedit sequence alignment editor (Hall 1999).

Data Analysis

Genotypic and allelic frequencies were calculated by direct counting. Deviation from Hardy–Weinberg equilibrium (HWE) were analyzed using a Chi-square test. Population genetic indexes including observed heterozygosity (Ho), expected heterozygosity (He) were calculated based on Allendorf dan Luikart (2007), and value of Polymorphism Information Content (PIC) was calculated based on Botstein et al. (1980).

RESULTS AND DISCUSSION

The Position of SNPs

In the present study, the position of three SNPs (*AKIRIN2:c.*188G>A*, *EDG1:c.-312A>G* and TTN:*g.231054C>T*) was confirmed using the current RefSeq as presented in Table 1. The position of SNPs of *AKIRIN2:c.*188G>A*, *EDG1:c.-312A>G* and *TTN:g.231054C>T* are relative to the base position of 62261408, 42039159 and 18060564, when the nucleotide numbering based on genomic reference sequences. The *EDG1:c.-312A>G* is also relative to a position of 166 downstream of the transcription initiation site in the 5' UTR (Yamada et al. 2009b). Nevertheless, the SNPs numbering in the present study still uses the same as described in the previous studies (*c.*188G>A* by Sasaki et al. 2009; *c.-312A>G* by Yamada et al. 2009b and *g.231054C>T* by Yamada et al. 2009a) to avoid confusion.

PCR-RFLP Product

The specific fragments of *AKIRIN2*, *EDG1* and *TTN* genes containing targeted SNPs were successfully amplified up to the size of 170 bp, 378 bp, 227 bp, respectively (Figure 1). These results are consistent with the result of Sasaki et al. (2009), Yamada et al. (2009b) and Yamada et al. (2009a) which use the same primer designs to amplify the *AKIRIN2*, *EDG1* and *TTN* genes, respectively.

In the present study, the genotyping was performed using the PCR-RFLP method by the restriction enzyme *Fok*I, *Msc*I, and *Hpy*CH4III for *AKIRIN2*, *EDG1*, and *TTN* genes, respectively. The genotypic band pattern of each gene and sequence chromatograms are shown in Figure 2. Digestion of PCR products of *AKIRIN2* in Bali cattle was detected in two genotypic band patterns involved *GG* (65 and 105 bp) and *AG* (65, 105 and 170 bp) genotypes; *EDG1* gene was only *AA* genotype (163 and 215 bp); while for *TTN* gene were *CC* (35 and 192 bp) and *CT* (35, 192 and 227 bp) genotypes. The genotypic findings in present study suggested that there were two alleles (*A* and *G* alleles) for *AKIRIN2*, one allele (*A* allele) for *EDG1* and two alleles (*C* and *T* alleles) for *TTN* genes in Bali cattle. These genotypic band patterns are in accordance with Kim et al. (2013) and Sasaki et al. (2009) study for *AKIRIN2* gene, by Sukegawa et al. (2010) and Tong et al. (2013) for *EDG1* gene. However, after checking the restriction site of *MscI* (ACT*GT) in targeted *TTN* sequence, the fragment size of C allele of *TTN* gene in present study (35 and 192 bp) is slightly different from Yamada et al. (2009a), where the fragment size of C allele resulted in 36 and 191 bp.

Genetic Polymorphism

The genotype and allele frequencies, observed heterozygosity (Ho), expected heterozygosity (He) and polymorphism information content (PIC) are shown in Table 2. The genotype of *AKIRIN2:c.*188G>A* detected in Bali cattle were the *GG* (99.2%) and *GA* (0.8%), while *AA* genotype was not detected. The *GG* genotype was found to be the most frequent in Bali cattle, but not in Japanese Black and Korean cattle where the *AG* genotype was the most frequent genotype (48.0 to 52.3% and 64.7 to 73.2%, respectively) (Sasaki et al. 2009; Watanabe et al. 2011; Kim et al. 2013; Sukegawa et al. 2014). The *G* allele was to be the predominant allele in Bali cattle (0.996). The very high frequency of *G* allele in the present study was also detected in Japanese Shorthorn (0.975 to 0.981), Holstein (0.790) and Brown Swiss cattle (0.787) (Watanabe et al. 2011). In contrast, the frequency of *G* allele tends to be less in Japanese Black cattle (0.262 to 0.568) (Sasaki et al. 2009; Watanabe et al. 2011; Sukegawa et al. 2014) and Japanese brown (0.389 to 0.430) (Watanabe et al. 2011).

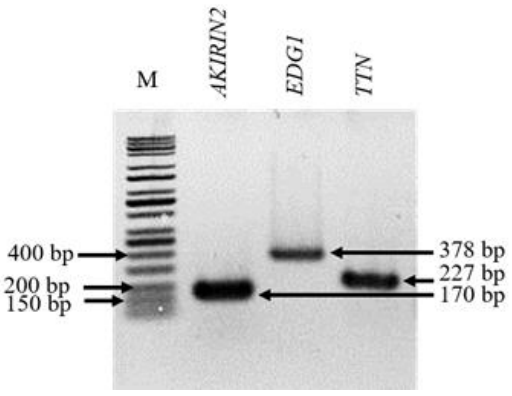

Figure 1. Amplicon size of *AKIRIN2* (170 bp), *EDG1* (378 bp) and *TTN* (227 bp) genes from Bali cattle DNA in a 1.5% (w/v) electrophoretic agarose gel. M = DNA ladder 100 bp.

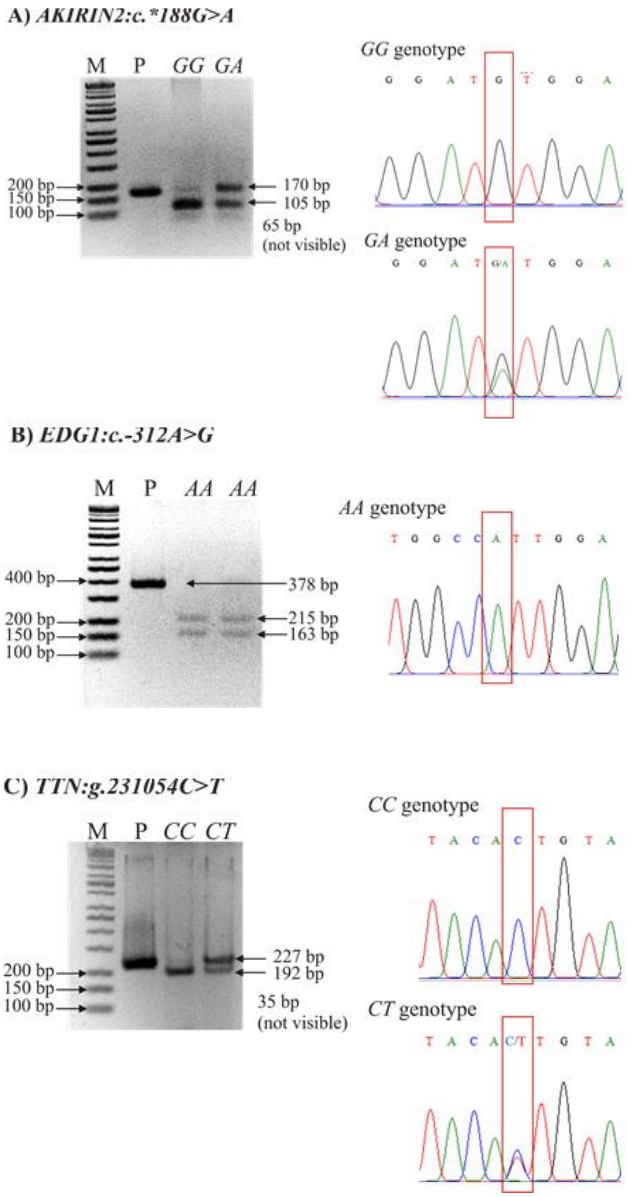

Figure 2. The genotypic band patterns by PCR-RFLP and chromatogram sequences of *AKIRIN2*, *EDG1* and *TTN* genes detected in Bali cattle. The arrows indicated the size of DNA fragments which separated on 3% agarose gel. M= DNA ladder 100 bp; P = PCR product of *AKIRIN2* (170 bp), *EDG1* (378 bp) and *TTN* (227 bp).

In the SNP of *EDG1:c.-312A>G*, all individuals of Bali cattle studied were detected to have *AA* genotype (100%) and consequently *A* allele was found to be fixed (1.000). In contrast, the *AA* genotype was considerably lower in Japanese Black cattle (16.6 to 35.4%) (Sukegawa et al. 2010; Tong 2013) and Hanwoo cattle (33.9%) (Shin and Chung, 2012) than in Bali cattle. However, the proportion of *A* and *G* were found to be balanced in both Japanese Black (0.411 to 0.581 and 0.419 to 0.589, respectively) (Watanabe et al. 2009; Sukegawa et al. 2010) and Hanwoo cattle (0.591 and 0.409, respectively) (Shin and Chung 2012). The high frequency of *A* allele in the present study was also detected in Japanese brown (0.953), Japanese Shorthorn (0.968), Holstein (0.996) and Brown Swiss (0.799) (Watanabe et al. 2009).

In the SNP of *TTN:g.231054C>T*, the observed genotype in Bali cattle was *CC* (99.2%) and *CT* (0.8%) genotypes. This finding indicates that *C* allele was the common allele found in Bali cattle as found in some cattle breeds raised in Japan (Yamada et al. 2009a; Watanabe et al. 2011). In addition, the distribution of the high frequency of *C* allele in the present study (0.996) tends to be similar as in Japanese Shorthorn (0.910), Holstein (1.000) and Brown Swiss (1.000) and higher than in Japanese Black (0.601 to 0.835) and Japanese Brown (0.724).

The He, Ho and PIC value of *AKIRIN2:c.*188G>A* and *TTN:g.231054C>T* were similar (0.008). Similar value of He and Ho reflects that these two markers are in HWE. In addition, based on the value of PIC value category (Botstein et al. 1980), the polymorphism of *AKIRIN2:c.*188G>A* and *TTN:g.231054C>T* were very low in Bali cattle and could be considered as monomorphic as well as for *EDG1:c.-312A>G*.

According to the previous studies, the A allele of *AKIRIN2*, the G allele of *EDG1* and the T allele of *TTN* genes were considered as the favorable alleles for high-marbling (Sasaki et al. 2009; Yamada et al. 2009a; Sukegawa et al. 2010; Shin and Chung 2012; Kim et al. 2013; Sukegawa et al. 2014; Chung 2015). Those all alleles are found to be high in Japanese Black cattle which have been subjected to a strong selection for high-marbling, but are found at very low or absent in Japanese Shorthorn, Holstein and Brown Swiss cattle that have not been selected for high-marbling (Watanabe et al. 2009; Watanabe et al. 2011). In the present study, the favorable alleles were also found at very low or absent in Bali cattle. This is possible because there has never been a selection program for high-marbling in the history of Bali cattle breeding in Indonesia. Watanabe et al. (2010) and Watanabe et al. (2011) stated that the strong selection pressure has led to selective sweep for favorable alleles that have a direct impact on marbling including of *AKIRIN2*, *EDG1*, and *TTN* gene. It may also happen in Korean cattle since the favorable alleles were found in high frequency.

The finding of very low or absent of the favorable alleles of *AKIRIN2*, *EDG1* and *TTN* genes in the present study provide a presumption that Bali cattle may not genetically potential as a high-marbling cattle breed. This presumption seems corroborated by several previous studies in Bali cattle. Anwar et al. (2017) revealed that the *T* allele of thyroglobulin gene (TG5) as favorable allele for marbling was not found in three different regions of pure-breeding of Bali cattle. In the study on marbling score using ultrasonic scanning method, Sri Rachma and Harada (2010) showed that the marbling score for Bali bulls at the age of 24 months was very low (0.17 to 0.20) (scale of 0 to 5 based on beef carcass grading standard of Japan). Likewise, by Jakaria et al. (2017), the marbling score for Bali bulls at the age of 6 years and Bali cows at the age of 3 years were only 3.80±0.84 and 4.50±1.05, respectively (the scale of 0 to 9 based on AUS-MEAT/MSA marbling reference standards).

Table 2. Genotype, allele frequencies, HWE test, Ho, He and PIC value of *AKIRIN2*, *EDG1* and *TTN* gene in Bali cattle

Gene	SNP position	n	Genotype frequencies (%)			Allele frequencies		HWE		Ho	He	PIC
								χ2test	χ2tab			
AKIRIN2	*c.*188G>A*	125	*GG*	*AG*	*AA*	*G*	*A*					
			99.2	0.8	0.0	0.996	0.004	0.002	3.841	0.008	0.008	0.008
EDG1	*c.-312A>G*	82	*AA*	*GA*	*GG*	*A*	*G*					
			100.0	0.0	0.0	1.000	0.000	-	-	-	-	-
TTN	*g.231054C>T*	125	*CC*	*CT*	*TT*	*C*	*T*					
			99.2	0.8	0.0	0.996	0.004	0.002	3.841	0.008	0.008	0.008

Note: n = number of animals; HWE = Hardy-Weinberg Equilibrium; χ2test <χ2tab (α=0.05) means that genotype frequency is in HWE

Oematan (2000), did not find any marbling in all carcass of Bali cattle evaluated that were treated using high energy rations. Furthermore, he stated that Bali cattle do not accumulate fat in intramuscular adipose but internal fat depots, especially around the kidneys. On the study of the histological structure and histomorphometry of muscle and intramuscular fat depots, Suwiti et al. (2015) showed that significantly different between Wagyu and Bali cattle. These evidences support the statement that marbling characteristic in cattle is dependent to breed of cattle (Gotoh et al. 2009; Duarte et al. 2013) and subsequently in the distribution of genotypes and alleles of the associated genes. In addition, Campbell et al. (2016) stated that any possible genetic differences in adipose tissue metabolism between *Bos taurus* and *Bos indicus* breed and may also exist in Bali cattle (*Bos javanicus*).

According to the result of the present study, it may be difficult and less efficient to obtain a high-marbling in Bali cattle when the selection using genetic marker of *AKIRIN2*, *EDG1*, and *TTN* gene is carried out within Bali cattle population without introgression of favorable alleles. Crossbreeding program may be used for those purposes. The Japanese Black, Japanese Brown or Hanwoo Korean cattle are the most promising breeds used in crossbreeding since they have been known to have favorable alleles in high frequency for high-marbling. However, it should be investigated further.

It could be concluded that the *A* allele of *AKIRIN2:c.*188G>A*, the *G* allele of *EDG1:c.-312A>G* and the *T* allele of *TTN: g.231054C>T* SNPs as the favorable alleles for high-marbling were found to be very low or absent in Bali cattle. Exploration of specific genes for high-marbling still needs to be investigated further or could use directed introgression breeding program by choosing the desired genotypes in selected individuals of certain cattle breeds into Bali cattle population.

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