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Expression of Myostatin Gene in Belgian Blue and Ongole Grade Crossbred Cattle

Winni Liani Daulay[1], Putri Indahningtyas[3], Cece Sumantri[2], and Jakaria[2]*

[1]Graduate School of Animal Science Faculty, IPB University, Bogor, 16680, Indonesia
[2]Department of Animal Production and Technology, Faculty of Animal Science, IPB University, Bogor, 16680, Indonesia
[3]Livestock Embryos Centre (LEC) Cipelang, Bogor, 16740, Indonesia

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* Corresponding author:
Telp. +62 816 4831 050
E-mail : jakaria@apps.ipb.ac.id

ABSTRACT

Investigating Myostatin (MSTN) as a potent inhibitor of skeletal muscle growth and development to produce excessive muscles is extremely essential for livestock breeding. This study aimed to analyze the expression of the MSTN gene and its relationships with genotype and phenotype (normal-muscled vs double-muscled) of Belgian Blue (BB) x Ongole Grade (PO) crossbred cattle. For that purpose, 12 animals from BB, PO, BB x PO F1, and BB x PO F2 cattle (3 animals each) raised at Balai Embrio Ternak (BET) Cipelang Bogor, West Java were used for blood sample collection. Genotyping analysis was performed using the PCR-RFLP method withprimer F: 5’-CTC TTC TTT CCT TTC CAT ACA GAC-3’ and R: 5’-AGG GGA AGA CCT TCC ATG TT-3’, while the MSTN gene expression was analyzed using the qPCR technique. As results, three genotypes: del.11/del.11, +/del.11, and +/+ were detected. The del.11/del.11 genotype, which showed a double-muscled phenotype was found in BB cattle and BB x PO F2 cattle. The +/del.11 genotype was found in BB x PO F1 cattle and BB x PO F2 cattle. The +/+ genotype, which showed a normal phenotype was only detected in PO cattle. There was a significant difference of the MSTN gene expression in the sampled animals among genotypes and between phenotypes (normal-muscled vs double muscled). The MSTN expression in animals with del.11/del.11 genotype was higher than that in animals with +/del11 and +/+ genotypes ($P<0.05$). Animals with +/+ genotype showed the lowest MSTN expression. It was concluded that double-muscled animals showed higher MSTN expression than normal-muscled animals.

Keywords: Cattle, Crossbreeding, MSTN gene, PCR-RFLP, q-PCR

Introduction

Myostatin (*MSTN*) is a member of the growth and differentiation factor superfamily (GDF-8), which is the sole inhibitor of skeletal muscle growth (Patel and Amthor, 2005). The inhibition of the *MSTN* activity can cause an excessive muscle growth, such as an increase in the number and diameter of muscle fibers (Zhang *et al.,* 2012). The bovine *MSTN* gene consists of three exons and two introns, of which the coding region encodes a protein with 375 amino acids (Jeanplong *et al.,* 2001).

Natural mutation in *MSTN* gene can produce double muscles in animals, consequent to loss of functional *myostatin* by disrupting several physiological processes involved in the creation and determination of the functional characteristics of muscle fibers (Cassar-Malek *et al.,* 2007). Many studies have been carried out by modifying the *MSTN* gene to obtain superior livestock that has a high percentage of carcass and meat quality. Naturally occurring mutations in the *MSTN* gene leading to excessive muscle build-up in mammals have been documented in sheep (Clop *et al.,* 2006; Boman *et al.,* 2009), dog (Osman *et al.,* 2021), and cattle (Kambadur *et al.,* 1997; McPherron and Lee, 1997).

The most renowned double muscle phenomenon is in Belgian Blue cattle, which were obtained from crosses between Holstein Friesian (FH) cattle and Shorthorn cattle, which have been developed in Belgium since 1850 (Purchas *et al.,* 1992). McPherron and Lee (1997) found the cause of double muscle in Belgian Blue cattle was due to a deletion of 11 nucleotide bases in exon 3 of the *MSTN* gene, while in Piedmontese cattle it was caused by a G–A transiton mutation at position 941 of the coding region in the *MSTN* gene that converts cysteine residues into tyrosine (Kambadur *et al.,* 1997).

The polimorphysm of the *MSTN* gene has been linked to increased growth traits and carcass in several cattle populations in various countries, including Bali cattle in Indonesia (Prihandini *et al.,* 2021). Khasanah *et al.* (2016) reported that the *myostatin* promoter gene was polymorphic in Bali cattle and there were 2 SNPs (g.-7799T>C and g.-7941C>T) associated with carcass quality. Other previous studies have also found the MSTN gene in cattle, including Qinchuan cattle (Zhang *et al.,* 2007), Angus cattle (Gill *et al.,* 2009), Nellore cattle (Grisolia *et al.,* 2009), Hanwoo cattle (Han *et al.*, 2012), and Marchigiana cattle (Sarti *et al.,* 2014).

The Belgian Blue cross-program with other cattle breeds as an attempt to increase cattle productivity has been carried out with Swiss Brown, Simmental, and Rendena cattle breeds (Tagliapietra *et al.,* 2018), Jersey dairy cow (Goni *et al.,* 2016), Hereford and Angus (Freetly *et al.,* 2011). Cross-breeding of beef cattle and dairy cows has a positive impact and produce several benefits (Weaber, 2015). Fundamentally, the goal of the cross-program is to obtain the effect of heterosis or hybrid vigor and to get the best combination of the two cross elders or races (Weaber, 2015). Belgian Blue (BB) cattle, which are double-muscled cattle from Belgium, were introduced to Indonesia in 2013 (in the form of embryos and semen) and began to be developed in 2015 at the Livestock Embryo Center (BET) Cipelang Bogor (Jakaria *et al.,* 2019). While the Ongole Grade cattle are one of the local Indonesian cattle breeds that have good adaptability in tropical environmental conditions with low feed quality (Romjali, 2018). The BB x PO cross was carried out to produce a generation of cattle that had a combination of superior traits from both parents. Agung *et al.* (2016) reported the F1 generation (Belgian Blue x FH and Belgian Blue x Sumba Ongole (SO)) had the MSTN gene in a heterozygous condition, thus providing scientific evidence that deletion of 11 bases in exon 3 of the MSTN gene is also exists or can be inherited. The evaluation of cross-breeding Belgian Blue cattle with Ongole Grade (PO) in the first generation (F1) that was conducted at LEC Cipelang Bogor showed a significant effect on increasing weaning weight and weight per year (Jakaria *et al.,* 2019). SNPs and indel 11-bp of *MSTN* genes associated with double-muscled phenotype in Belgian Blue crossbred with PO cattle were also found (Jakaria *et al.,* 2021). The analysis of MSTN gene expression at the mRNA transcript level to identify the role of the MSTN gene in producing the double-muscled trait has been reported (Kambadur *et al.*, 1997; Oldham *et al.,* 2001) that MSTN mRNA was higher in double-muscled than normal cattle. The aim of this study was to analyze the expression of the *MSTN* gene in the first and the second generation of Belgian Blue x Ongole Grade crossbreed to determine the involvement of *MSTN* gene in producing the double-muscled cattle breed.

Materials and Methods

D blood collection

All procedures involving animals were approved by the Livestock Embryos Center (LEC) in Cipelang, Bogor, Indonesia. The procedures for blood collection also followed the principles of animal welfare. Blood samples were collected from a total of 12 individual animals including *Belgian Blue cattle (n=3), Ongole Grade (PO) (n=3), F1 offspring (n=3), and F2 offspring (n=3).* F1 was the first generation of individual crossbred (BB × PO) cattle (*B. Taurus* × *B. Indicus*) with blood composition of 50% BB and 50% PO, while the second generation crossbred (F2) had a blood composition of 75% BB and 25% PO.

Blood samples were taken from coccygeal vein using multi Venoject needle with 5 mL vacutainer tubes contain EDTA. The blood samples were divided into two parts for DNA and RNA analysis. The blood samples used for RNA analysis were put in a tube 2 mL and immediately stored in a liquid nitrogen tube (temperature -81^{o}C) before being used for further analysis.

Amplification and genotyping of *MSTN* gene

Genomic DNA was isolated from whole blood samples using the modified Geneaid^{TM} Kit DNA extraction protocol. A pair of primers were used to amplify part of *MSTN* gene in exon 3. The forward primer: 5'-CTC TTC TTT CCT TTC CAT ACA GAC-3' and the reverse primer: 5'-AGG GGA AGA CCT TCC ATG TT-3' had a product length of 451 bp (Jakaria *et al.*, 2021). Amplification condition of PCR consisted of predenaturation at 95°C for 5 min, followed by denaturation at 95°C for 10 s, annealing at 60°C for 20 s, extension at 72°C for 30s and final extension at 72°C for 5 min. PCR Premix in tube 0.2 mL were made of a mixture consisting of 0.6 µL of primer, 12.5 µL of MyTaq HS RedMix, 9.9 µL of nuclease free water (NFW) and 2 µL of DNA samples. The *MSTN* gene was genotyped using enzymes *NmuCI* (*Tsp45I*) (Jakaria *et al.*, 2021) and R buffer by PCR-RFLP for 4 h at 37^{o}C. The digested product were separated using 2% agarose gel with current strength of 100 volt for 35 min and documented using a UV transillluminator (AlphaImager; Alpha Innotech, CA, USA).

Analysis of *MSTN* gene expression

Primer. The primers used in this experiment were picked from National Center for Biotechnology Information (NCBI) referring to accession number of each gene listed in Table 1. The primer length was determined using the Primer 3 program. The primers used for *MSTN* gene expression were determined and analyzed using the Multiple Primer Analyzer and Primer Stat programs. The β-actin gene was used as a housekeeping gene.

RNA isolation. Blood samples which were preserved at -81°C for five days before were isolated using the Qiagen^{TM} RNeasy fibrous tissue mini kit with a modified procedure. The thawed blood sample was then added with 1:1 PBS into a tube 2 mL. Samples were centrifuged for 10 min at 10,000 rpm. The supernatant was discarded, and the washing process was repeated three times using PBS. RLT buffer in the amount of 800 l was added, homogenized, and incubated at room temperature for seven minutes. The solution was homogenized using a 1 cc syringe with a needle bent in a zigzag form, then incubated at room temperature for 5 minutes. The solution was homogenized with 800 l of 70% ethanol until it was transparent. The solution was placed in the RNeasy Spin Column and centrifuged at 9,000 rpm for 1 minute. The filtrate was discarded after centrifugation for 1 minute at 8,000 rpm with 350 l RW1 buffer. The DNAse incubation mix was added to the spin column tube and the solution was incubated for 15 minutes. RW1 Buffer was added and centrifuged again. Then, 500 l of RPE buffer was added and centrifuged. This process is repeated twice. Furthermore, 30 l of RNAse-free water was added to a 1.5 mL tube that had been packed with a RNAsy spin column, and the sample was incubated for 1 minute at room temperature before centrifugation at 8,000 rpm for 1 minute. The isolated RNA was stored in the freezer at -81°C. RNA quantification was carried out using a Nanodrop Spectrophotometer.

Reverse Transcriptase cDNA. Reverse Transcriptase cDNA was carried out using cDNA synthesis Kit (Toyobo). The total RNA was diluted to 50 ng. 2 µL of total RNA was distributed into 0.2 mL tubes followed by the addition of 2 µL of 4x DNMM and 5 µL of NFW (nuclease free water) then homogenized using vortex and incubated at 37°C for 5 min. Furthermore, 2 µL of 5x RTMM was added and incubated using thermocycler machine Applied Biosystems GeneAmp PCR System 9700 (Thermo Fisher Scientific, Inc., USA) at 37°C for 15 min, 50°C for 5 min and at 98°C for 5 min. Finally cDNA can be stored at -20°C.

Real time q-PCR. Complementary DNA (cDNA) was used for *MSTN* gene expression quantification using real time PCR machine (AG qTower 4 channel Analytic Jena engine, Germany). qRT-PCR was performed using the SYBR green select master kit (Applied Biosystem, USA). The total reaction volume was 10 µL including 5 µL of SYBR green select master kit, 0.5 µL of each forward primer and reverse, 1 µL of cDNA and 3 µL of NFW (nuclease free water). Amplification condition of PCR consisted of predenaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 55 °C for 20 s, extension at 72°C for 30 s and final extension at 72°C for 5 min. *β-Actin* gene was used as a housekeeping gene to normalize the RT-PCR efficiency.

Statistical analysis

All data were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Scmittgen, 2001). The following formula was used to measure the relative change of gene expression of MSTN gene from tested group to control group compared to the housekeeping gene: ΔΔCT = (average Ct_{MSTN} in the tested group - average $Ct_{\beta\text{-}actin}$ in the tested group) - (average Ct_{MSTN} in the control group - average $Ct_{\beta\text{-}actin}$ in the control group). The +/+ genotypes and normal-muscled phenotypes were appointed as a control group. Statistical comparisons of MSTN gene expression among different genotypes and phenotypes of cattle breeds were determined by the Student t test and p<0.05 was regarded as statistically significant (Minitab® 18 Software). The mathematics model was (Kim, 2015):

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

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

Where:

$\bar{x}_1$ = the average of *MSTN* gene expression of genotype 1 or double-muscled phenotype

$\bar{x}_2$ = the average of *MSTN* gene expression of genotype 2 or normal-muscled phenotype

n_1 = Number of individuals of genotype 1 or double-muscled phenotype

n_2 = Number of individuals of genotype 2 or normal-muscled phenotype

s = the combined of standard deviation

Results and Discussion

Genotyping of *MSTN* gene

The MSTN gene in Belgian Blue, PO and BB x PO crossbred was successfully amplified with product length 451 bp (Figure 1). Agarose gels exhibited bright single bands without smear at the expected size. The results showed that the amplified fragment had a high level of specificity, indicating that RFLP analysis could be carried out directly.

The PCR-RFLP technique using MSTN|*NmuC*I (Tsp45I) was successfully identified the difference between double-muscled and normal appearance in Belgian Blue, Ongole Grade, and BB x PO crossbred cattle (Figure 2). The del.11/del.11 genotype (350bp and 90bp) showed the double-muscled phenotype was found in Belgian Blue and BB x PO F2 cattle. The +/+ genotype (451bp) was found in all of the PO cattle. The heterozygous genotype (+/del.11) (451 bp, 350 bp and 90 bp) was found in F1 and two F2 crossbred with normal phenotype (Figure 2).

The inheritance pattern of allele + and allele del.11 or +/+ genotype, +/del.11 and del.11/del.11 genotype has been illustrated in Figure 3. The heterozygous F1 offspring was backcrossed with double-muscled Belgian Blue, resulting in a double-muscled and normal F2 offspring.

Based on the results of the genotyping, it was determined that the inheritance pattern of the double-muscled trait was expressed in a recessive homozygous state. Where the double-muscled

Table 1. The primers were used for *MSTN* gene expression using qPCR method

Gene	Primer sequences^1	Product size (bp)	Annealing temperature (℃)
*MSTN**	F: 5'-GAGAGATGCCAGCAGTGACG-'3 R: 5'-CCTGTCAAGACTCCTGCGAC-'3	213	55
β-actin**	F: 5'-GGACTTCGAGCAGGAGATGG-'3 R: 5'-GCGGCATTCACGAAACTACC-'3	172	55

$^{1)}$Hamny (2020); *) AB076403; **) NM_173979.3 ; F: *forward* and R: *reverse.*

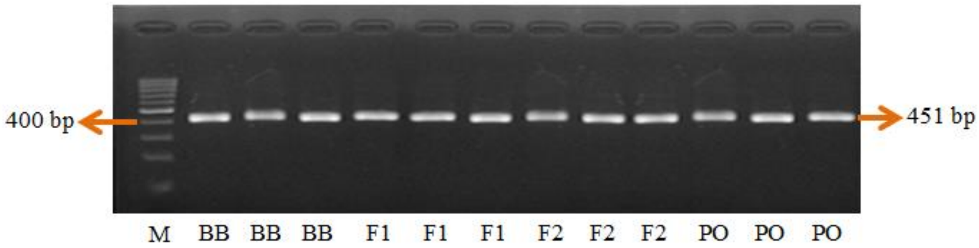

Figure 1. The electrophoresis of PCR product of *MSTN* gene in 1,5% of agarose gel (M: Marker; BB: Belgian Blue; PO: Peranakan ongole; F1: 50% BB x 50% PO; F2: 75% BB x 25% PO).

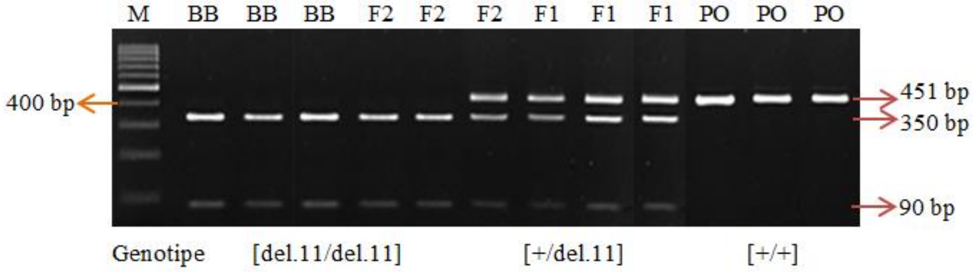

Figure 2. The electrophoresis of PCR-RFLP of *MSTN* ǀ*NmuCI* (*Tsp451*) gene in 2% of agarose gel (M: Marker; BB: Belgian Blue; PO: Peranakan Ongole; F1: 50%BB x 50%PO; F2: 75% BB x 25% PO).

trait did not appear in a heterozygous condition in Belgian Blue and PO crossbred which were grouped into the normal phenotype. It is similar to the double-muscled phenomenon in Belgian Blue and Piedmontese cattle were inherited as recessive (Kambadur *et al.,* 1997; McPherron and Lee, 1997). The double-muscled Marchiagiana cattle were also found in homozygous condition (Marchitelli *et al.,* 2003). In other animals, Boman *et al.* (2009) found a deletion of 1 bp at position c.960delG of the *MSTN* gene in Norwegian white sheep with homozygous condition where the phenotype was characterized by a high carcass conformation class and low fat class. Osman *et al.* (2021) found SNP c.18 G>T and SNP c.241 T>C in the *MSTN* gene in Egyptian sheep were associated with growth traits where the GG genotype showed a higher birth weight and the TT genotype was associated with the average daily gain of sheep.

The phenomenon of double muscles in cattle, especially in Belgian Blue crosses with Indonesian local cattle breeds is important for better breeding strategies in the future. The appearance of the double-muscled phenotype in the second generation of the BB x PO crossbreed was demonstrated to be an effective approach to boosting muscle growth in livestock production.

In addition, there are several problems found in double-muscled cattle, including decreased female fertility, lower offsprings viability, and deferred in sexual maturation (Bellinge *et al.,* 2005; Arthur, 1995). Kolkman *et al.* (2010) also reported high cases of dystocia in the population of double-muscled Belgian Blue cattle, reaching 81.63% or 120 out of 147 calves born by caesarean section due to a greater shoulder width and heart girth. Short *et al.* (2002) reported a decreased pelvic area in double-muscled Piedmontese cattle. Pelvic opening of double-muscled dams was 10 and 6% lower than in normal-muscled Charolais (Arthur *et al.,* 1988) so that the occurrence of dystocia and perinatal mortality was higher in double-muscled cattle. Interestingly, Heterozygous animals did not show any increase in calving difficulty compared to normal animals (Arthur *et al.,* 1988; Blasi *et al.,* 1991; Kišacová *et al.,* 2009). Hopefully, the discovery of genetic markers for the *MSTN* gene (Jakaria *et al.*, 2021) in the crossbreeding program of Belgian Blue cattle with PO or other Indonesian local cattle can reduce the risk of dystocia cases (calving difficulty).

Identification of *MSTN* gene polymorphism and its association with growth traits will provide convenience for breeders to select individual livestock that are considered superior so that they can assist livestock producers in developing breeding strategies to optimize livestock potential. Through various approaches, the exploitation of *MSTN* gene mutations can provide significant benefits for several livestock industries (Ahad *et al.*, 2017).

MSTN gene expression

The mRNA transcription level of *the MSTN* gene was measured via the qRT-PCR technique and the results were performed in Table 2. The *MSTN* mRNA level in del.11/del.11 genotype was lower than +/del.11 ($P<0.05$). However, The *MSTN* mRNA levels between the del.11/del.11 genotype and the +/+ genotype were not significantly different. Likewise, in the +/del.11 genotype and the +/+ genotype, there was no difference in the levels of *MSTN* mRNA in either ($P>0.05$) (Table 3). The statistical test results were not significantly different due to the very limited number of samples. The total of samples analyzed was 12 individuals, but only 9 samples were successfully isolated for RNA for qRT-PCR analysis. The *MSTN* gene expression between phenotypes showed a significant difference, where the double-muscled phenotype had a lower *MSTN* mRNA level than in normal-muscled phenotype ($P<0.05$). The *MSTN* gene expression in BB, PO and their crossbred with different genotypes and phenotypes are presented in Figure 4. The qPCR results indicated that the *MSTN* mRNA transcript level in homozygous double-muscled cattle was substantially decreased compared to heterozygous and homozygous normal-muscled cattle. The heterozygous individuals also encountered a decreased *MSTN* mRNA level compared to normal cattle.

Myostatin (*MSTN*) is the sole inhibitor of skeletal muscle growth and development (Patel and Amthor, 2005). Loss of myostatin fuction increased the diameter and number muscle mass (Zhang *et al.,* 2012). *MSTN*-knockout mice have an incredible increase in skeletal muscle mass and a significantly decreased fat percentage

Table 2. The analysis of qRT-PCR of *MSTN* gene in cattle

Breed	Phenotype	Genotype	ΔCT	Mean ΔCT	ΔΔCT	$2^{-\Delta\Delta CT}$
Belgian Blue	Double muscle	del.11/del.11	9.05	9.47 ± 0.59	5,79 ± 0.01	0,02 ± 0.01
F2 (75%BB x 25%PO)	Double muscle		9.89			
F2 (75%BB x 25%PO)	Normal muscle	+/del.11	7.14	5.67 ± 1.01	1,99 ± 0.15	0,3 ± 0.15
F1 (50%BB x 50%PO)	Normal muscle		5.46			
F1 (50%BB x 50%PO)	Normal muscle		4.84			
F1 (50%BB x 50%PO)	Normal muscle		5.25			
PO	Normal muscle	+/+	4.18	3.68 ± 0.78	0.00 ± 0.65	1.00 ± 0.65
PO	Normal muscle		2.79			
PO	Normal muscle		4.08			

Table 3. The differences of *MSTN* gene based on genotype and phenotype

Genotype	*t*-test (P-value)
del.11/del.11 vs +/del	0.035*
del.11/del.11 vs +/+	0.101
+/del vs +/+	0.166
Phenotype	*t*-test (P-value)
Double muscle vs Normal muscle	0.030*

(*) significant at α=5%.

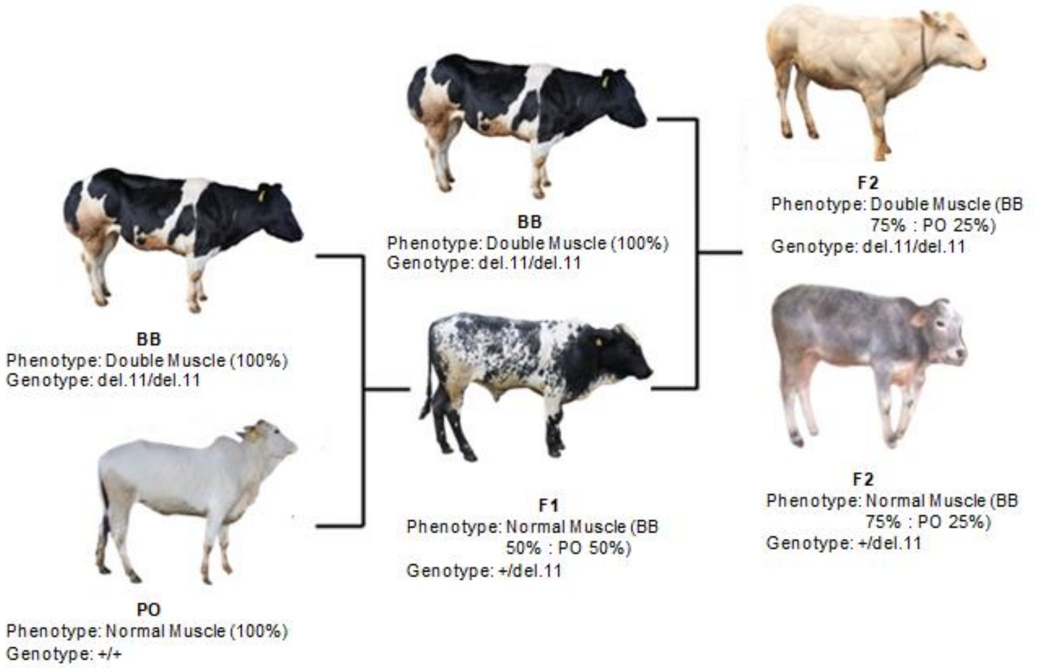

Figure 3. The inheritance of allele + and allele del.11 in Belgian Blue and PO crossbreed.

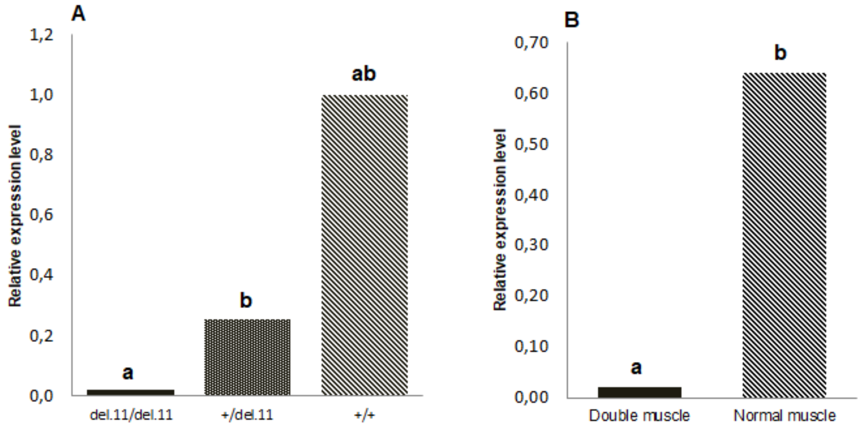

Figure 4. *MSTN* gene expressions (Different A genotype, Different B phenotype). ^{a,b} indicate significant differences (P<0.05) and ^{ab} indicates no significant difference (P>0.05).

compared to the wild-type (McPherron *et al.,* 1997). The decreased of mRNA transcription level of mutant *MSTN* in double-muscled cattle suggests that mutant *MSTN* gene can not be successfully transcripted and ultimately produce a disrupted myostatin protein due to the 11-bp deletion in Exon 3. When the structure of the myostatin function is inhibited, resulting in the changes of *CDK2* and *P21* expression levels which are effectively encourage the proliferation of bovine fibroblast cells (Gao *et al.,* 2014). In the double-muscle Javanese cattle, myostatin inhibition can reduce the GLUT4 mRNA to produce the excessive muscle relative to normal-muscled cattle may be due to their greater use of glucose (Takahashi *et al.,* 2014). Hu *et al.* (2013) reported that the *MSTN* gene expression was significantly prevented in transgenic sheep, leading to a faster increase in body weight than in control sheep. Qian *et al.* (2015) showed that *MSTN* gene expression was not detectable in double-muscled Meishan pigs containing a segment with a 193 bp deletion in exon 2 of the *MSTN* gene compared to normal pigs.

On the other hand, Kambadur *et al.* (1997) reported that there was no difference in *MSTN* gene expression between double-muscled Belgian Blue cattle compared to normal muscle using the RT-PCR technique. Evaluation of protein changes in cDNA sequences in double-muscled cattle revealed an 11 bp deletion resulting in the loss of three amino acids (275, 276, and 277) and the presence of a frameshift mutation after amino acid 274.

Frameshift mutations are caused by insertions or deletions that disrupt the DNA sequence. After the insertion or deletion point, each mRNA created from a modified DNA sequence will be read out of the target fragment, resulting in a different protein than usual (Pelley, 2012). The same phenomenon was reported by Boman *et al.* (2009) in Norwegian sheep, which had an increase in muscle mass due to a frameshift mutation in the *MSTN* gene, which caused the formation of a premature stop codon and eventually formed the imperfect protein as a normal, eventually reducing the *MSTN* gene function.

Conclusions

In Belgian blue cattle, PO cattle, and their crosses, *MSTN* gene expression was variable in different genotypes and phenotypes. In the examined cattle, the *MSTN* gene expression in del.11/del.11 genotype (double-muscled) decreased compared with heterozygous (+/del.11) and +/+ genotype. Similarly, the *MSTN* gene expression was lower in the double-muscled phenotype than in the normal-muscled phenotype.

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