MYOSTATIN GENE POLYMORPHISMS AMONG BELGIAN BLUE CROSSBREDS IN SMALLHOLDER FARMS IN MALAYSIA

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ABSTRACT. Beef cattle's growth phenotype is linked to their productive cycles determined by the functional group of genes. The candidate gene approach examines genes with subtle effects involved in underlying growth physiological pathways. The double-muscling trait is associated with the highly muscular double-muscled continental breed Belgian Blue caused by polymorphisms specifically myostatin gene mutations (deletions). The trait is integrated into tropical beef cattle herds, resulting in excellent economic value and meat production. The Belgian Blue breed and its crossbreds frequently obtain dressing percentages greater than 60 %, making it a feasible option for farmers who wish to produce leaner meat. Polymerase chain reaction (PCR) and Sanger sequencing analysis were utilized in this study. The objective is to detect specific mutations in Belgian Blue crossbred offspring from Malaysian smallholder beef cattle farming, confirming the genetic basis of enhanced phenotypes and growth projections. 11-base pair (bp), 10-bp, and 6-bp nucleotide deletions were discovered among the Belgian Blue crossbred offspring at various nucleotide (nt) locations. All findings were validated with a beef cattle double-muscling-related literature review. The results offer valuable insights for smallholder farmers, policymakers, and researchers, allowing informed decisions about double-muscling traits and their genetic material utilization in tropical and Malaysian contexts.

Keywords: beef cattle, Belgian Blue crossbreds, double muscling, myostatin, mutations

INTRODUCTION

Genes and their polymorphisms profoundly impact the efficiency of cattle fattening, growth projection, and consequently, the economic value of slaughter. Although not universally applicable to all cattle breeds, the influence of the studied markers on various traits can be extrapolated to specific growth studies (Ceccobelli *et al*., 2022). The growth phenotype of beef cattle, which encompasses hereditary genetic characteristics and environmental influences, is directly linked to their productive cycles (Mendonça *et al*., 2019). Using conventional selection techniques to improve growth metrics is challenging because of polygenic control and limited heredity (Favero *et al*., 2019). Apart from understanding cellular development, knowledge of genes and

chromosomal regions associated with desirable overall growth characteristics can be highly beneficial when determining the breeding value of offspring (Ceccobelli *et al*., 2022). Evaluating these characteristics is both expensive and time-consuming. The candidate gene approach examines the genes that may be involved in the physiological pathways that control growth in order to determine the connection between a trait of interest and known genes (Naserkheil *et al*., 2022). This method can effectively identify loci with even subtle effects if the candidate gene is causal. Among the genes that receive significant attention from researchers and beef cattle producers is myostatin, a member of the transforming growth factor (TGF) superfamily. Studies have shown that this gene family has a significant impact on postnatal skeletal muscle mass and growth (Esmailizadeh *et al*., 2008; McPherron, 2012), acting through autocrine and paracrine signalling mechanisms (Chen *et al*., 2021). Furthermore, the TGF superfamily is a group of multifunctional proteins and highly conserved across mammals (Adoligbe *et al*., 2012; Kambadur *et al*., 1997). The myostatin gene in mammals has a relatively conserved structure with two introns and three exons that code for a 375-residue protein (Chen *et al*., 2021).

Over the past 190 years, the double-muscling trait in cattle has been observed in various breeds. It appears to be inherited as a single major autosomal locus, with several modifiers influencing the expression of the phenotype, primarily due to the incomplete penetrance of the myostatin gene (McPherron, 2012). This genetic condition is also referred to as a polymorphism or mutation of the gene (Aiello *et al*., 2018). In a study by Charlier *et al.* (1995), a linkage analysis was conducted using a panel of microsatellite markers that covered the entire bovine genome. It successfully localized the autosomal muscular hypertrophy (mh) locus of the myostatin gene to bovine chromosome 2. Subsequently, Grobet *et al*. (1998) determined the complete coding sequence of the myostatin gene from 32 cattle belonging to ten European breeds with remarkable muscular development. That study identified several polymorphisms within the gene that were expected to disrupt myostatin function. These polymorphisms included nt821 (del11), F94L, Q204X, E226X, nt374-51(T-C), nt374-16(del11), nt414(C-T), nt419 (del7-ins10), nt748-78(del11), and C313Y. In the United Kingdom (UK) and Ireland, the four most common myostatin mutant variants observed among cattle are nt821 (del11) (found in Belgian Blue), F94L (Limousin and Aubrac), Q204X (Charolais), and E226X (Beef Shorthorn breeds) (Nigel, 2023; Vankan *et al*., 2010). Additionally, there are at least six myostatin gene variants associated with the double-muscling phenotype: nt821 (del11), nt419 (del7-ins10), nt610 (CT), nt676 (GT), nt874 (GA), and nt938 (GA) (Grobet *et al*., 1998; Kambadur *et al*., 1997; McPherron & Lee, 1997). The 11-base pair (bp) deletion mutation (nt821 (del11)) of the myostatin gene is particularly significant and strongly correlated with the double-muscling trait. This mutation, commonly found in the Belgian Blue breed but not limited to it, results in protein truncation and the introduction of a premature stop codon, disrupting muscle regulation and leading to muscular hypertrophy and hyperplasia (Gill *et al*., 2009). Under normal circumstances, myostatin binds to the activin type IIB receptor (ACVR2B) on the surface of muscle cells, activating downstream signalling pathways that inhibit muscle hypertrophy. However, in double-muscled cattle, the absence or reduced activity of myostatin due to gene mutations disrupts this signalling pathway, resulting in increased muscle mass (Kambadur *et al*., 1997).

Belgian Blue, a Continental breed, is renowned for its characteristic of double-muscling, which has been documented in previous studies (Arthur, 1995; Fiems, 2012; Grobet *et al*., 1997). Initially, it was believed that the double-muscling trait and mutated myostatin gene could only be inherited in purebred double-muscled cattle (homozygous recessive or mh/mh) (Agrawal *et al*., 2017; Charlier *et al*., 1995; Kambadur *et al*., 1997). Studies by Jakaria *et al*. (2021) and Agung and Said (2014) challenged this notion. They showed that Belgian Blue crossbred offspring (heterozygous or mh/+) could inherit a single copy of the mutated myostatin gene and exhibit higher weight compared to normal-muscled breeds (Agung *et al*., 2016). It has been established that beef cattle with the double-muscling trait yield more meat than other breeds (Fiems, 2012). These cattle exhibit 20-25 % (Ceccobelli *et al*., 2022; Grobet *et al*., 1997; McPherron & Lee, 1997) or even up to 28 % (Fiems *et al*., 2003) more muscle mass than normal-muscled cattle. Research indicates that double-muscled cattle carcasses contain 20-130 % more lean meat and 30-50 % less fat than normal-muscled bulls (Rollins *et al*., 1980). With increasing health consciousness, western beef producers aim to provide modern lean or organic red meat with minimal visible fat content (Tey *et al*., 2008). Consequently, raising the Belgian Blue breed offers a viable solution for producers seeking to produce leaner meat. It has been reported that Belgian Blue cattle often achieve dressing percentages exceeding 60 % (Purchas *et al*., 1992). Therefore, incorporating the genetic traits of Belgian Blue into beef cattle in tropical regions holds potential advantages, leading to increased economic value and beef output. If these superior traits could be successfully integrated into the offspring of tropical crossbred Belgian Blue cattle and reared within the available resources of these regions, it would not be surprising. The Belgian Blue breed has played a significant role in maintaining and increasing the SSL (self-sufficiency level) of beef supply in Belgium, reaching as high as 157 % in the 2000s (Fiems *et al*., 2003).

Various methods are commonly employed to detect gene polymorphism, including polymerase chain reaction (PCR) with agarose gel electrophoresis documentation, quantitative real-time PCR, PCR-restricted fragment length polymorphism (RFLP), Sanger sequencing, and Northern analysis (Ciepłoch *et al*., 2017). PCR cycles selectively amplify the myostatin gene from genomic DNA samples, facilitating subsequent analysis (Food and Agriculture Organization of the United Nations [FAO], 2011). Genomic DNA extracted from the samples serves as a template for PCR amplification, using specific primers designed to target the candidate gene (Kadri, 2019). Following PCR amplification, sequencing analysis, typically performed using Sanger sequencing, allows for the determination of specific polymorphisms in the candidate gene (Jakaria *et al*., 2021). Sanger sequencing is a technique that provides detailed information on the nucleotide sequence of the amplified DNA fragment, enabling the identification of specific mutations or genetic variants within the myostatin gene (Gao *et al*., 2007). As the "gold standard" in sequencing technology, Sanger sequencing utilizes the chain-termination method to accurately determine the identity and order of the four nucleotide bases in a DNA segment. It offers high accuracy, long-read capabilities, and versatility for various applications across multiple research fields (Fisher, 2003).

This study aims to detect the polymorphisms for specific mutations (deletions) present in the Belgian Blue crossbred offspring born and raised in Malaysian smallholder beef cattle farming. These mutations confirm the genetic

basis underlying the enhanced phenotype and growth of the offspring. Moreover, it evaluates the effectiveness of introducing the double-muscling trait into the local beef cattle population. The results of this study are useful for smallholder farmers, policymakers, and researchers, enabling them to make informed decisions regarding the utilization of genetic material from the Belgian Blue breed in tropical and Malaysian contexts.

MATERIALS AND METHODS

Polymerase Chain Reaction (PCR)

Blood Sampling, DNA Extraction, and Purification

Seventeen (n=17) male and eleven (n=11) female offspring were selected randomly from the population of Belgian Blue crossbred (*Bos taurus x Bos indicus*) offspring born and raised in Kuala Terengganu, Terengganu (5 °19'48.72"N, 103 °08'26.88"E) smallholder farms. These cattle were selected for blood sampling at the age of 180 days (6 months) based on their highest recorded weight. The choice of the 6-month age was made because, at this stage, the offspring were considered sufficiently developed in terms of musculature, primarily influenced by genetic factors and the dam's milk, while minimizing the impact of environmental factors. The offspring were born from an artificial insemination program that involved local cows of local indigenous breed (*Bos indicus*) which was Kedah-Kelantan, inseminated with Belgian Blue pure-breed frozen semen (*Bos taurus*). The study obtained approval from the Department of Veterinary Services, Malaysia (Ref: JPV.BPI.600-1/7/1), adhering to principles of animal welfare. Blood samples were collected from the selected cattle offspring through jugular vein puncture using 10 ml vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) at pH 8 as an anticoagulant. Each tube was appropriately labelled with the identification number of the respective offspring and placed in a 5 L Coleman cooler box with ice packs or crushed ice for transportation to the laboratory. Upon arrival at the laboratory, the samples were promptly stored at -20 °C.

Genomic DNA was extracted from thawed whole blood samples using the commercial QIAmp DNA kit (QIAGEN, Germantown, USA). The extraction process began with 20 µl of Qiagen proteinase K added to a 1.5 ml microcentrifuge tube. Then, 200 µl of the blood sample was combined with the proteinase K and mixed using a vortex shaker for 15 seconds. The mixture was incubated in a water bath at 56 °C for 10 minutes. Following this, 200 µl of ethanol (96-100 %) was added to the sample and vortexed for 15 seconds. The resulting mixture was carefully transferred to a QIAamp spin column placed in a 2 ml collection tube, ensuring that the column's rim was not wetted, and the lid was securely closed. The tube was centrifuged for one minute at 6,000 g (8,000 rpm), and the spin column was then transferred to a fresh collection tube while discarding the filtrate-containing tube. The cap of the spin column was removed, and 500 µl of buffer AW1 was added without wetting the column's rim. After centrifugation at 8,000 rpm for one minute, the QIAamp spin column was moved to a clean 2 ml collection tube, while the filtrate tube was discarded. The cover of the spin column was carefully opened, and 500 µl of buffer AW2 was added. The tube was then centrifuged at 20,000 rpm for three minutes. Finally, the lid of the spin column was opened, and 200 µl of buffer AE was added to elute the DNA. The QIAamp spin column was discarded, and the eluted DNA was stored at -20 °C for long-term preservation.

The quality and quantity of the extracted DNA were assessed using spectrophotometry

(NanoDrop, Thermo Fisher Scientific Inc., Waltham, MA, USA). To calibrate the NanoDrop, a 10 mm route length standard glass cuvette was filled with 500 µl of water, and blank readings were taken at 260 nm and 280 nm. For the DNA samples, 1 µl of the extracted DNA was dropped on the glass slide and the optical densities (ODs) or absorbance at 260 nm and 280 nm were measured. The A260/280 ratio was then determined to assess the purity of the DNA sample.

DNA Amplification

The primers utilized in this experiment were designed based on previous research conducted by Agung *et al*. (2016) (Table 1) to amplify exon 3 of chromosome 2 of the myostatin gene. The primer design also considered the reference sequences for *Bos taurus* (GenBank Accession No. AF320998.1) and Bos indicus (GenBank Accession No. AY794986.1) obtained from the National Center for Biotechnology Information (NCBI, 2023). For the PCR reaction, a total volume of 50 µL was prepared consisting of 25 µL of GoTaq® Green Master Mix (Promega Corporation, Madison, USA), which contained 1 µL each of forward and reverse primers, 1 µL of the DNA template, and 22 µL of nuclease-free water. The reaction mixture was then transferred into a sterile 0.2 ml PCR tube and thoroughly mixed using a vortex shaker. The PCR reaction was carried out using the Bio-Rad T100 Thermocycler (Bio-Rad Laboratories, Inc., California, USA) with the following thermocycling conditions: an initial denaturation step at 95 °C for 2 minutes (1 cycle), denaturation at 95 °C for 30 seconds (30 cycles), annealing at 52.5 °C for 30 seconds (30 cycles), extension at 72 °C for 1 minute (30 cycles), final elongation at 72 °C for 5 minutes (1 cycle) following the completion of the previous 30 cycles, and cooling at 4 °C for a flexible duration. Troubleshooting for the PCR thermocycling step was performed according to the guidelines provided by Promega manufacturer's guide with minor modifications (Lorenz, 2012).

Table 1. The product size, forward, and reverse primer sequence of the myostatin gene used in the study.

Candidate gene	Product size (bp)	Forward primer	Reverse primer	Reference
Myostatin (GDF8 @ MSTN)	660	5'- GGAAGAATCAAGCCTAGTGT-3'	5'- GCTTGTGCTTAAGTGACTGT-3'	(Agung et al., 2016)

Gel Electrophoresis Documentation

The amplified PCR products were further mixed with 1 µL of Promega DiamondTM Nucleic Acid Dye (Promega Corporation, Madison, USA) in a well of 2 % agarose gel. The Promega 100bp DNA ladder was also included in the first well for size reference. The amplification products were visualized using the Bio-Rad wide electrophoresis horizontal tank with a power pack (Bio-Rad Laboratories, Inc., California, USA) at 90 volts for 65 minutes. Subsequently, the amplified bands were captured and documented using the Bio-Rad Gel Doc XR+ System (Bio-Rad Laboratories, Inc., California, USA) under the built-in ultraviolet light.

Sanger Sequencing Analysis

The final step in identifying the amplified nucleotide sequence is Sanger sequencing. The PCR products obtained from the Belgian Blue

crossbred offspring were sent to Apical Scientific Sdn. Bhd. laboratory in Malaysia for sequencing analysis. The sequencing was performed using an ABI PRISM 3730XL Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, USA) with the PCR products serving as templates. Standard pairwise comparisons of nucleotide homology were conducted using the GenBank search (NCBI, 2023) and the Basic Local Alignment Search Tool (BLAST) database (BLAST, 2023) to compare the obtained sequences. Additionally, sequence alignment was performed using Molecular Evolutionary Genetic Analysis (MEGA) (Pennsylvania, USA) version 11.0 for Windows. This analysis aimed to identify any mutations (deletions) in the nucleotide sequence by comparing it to the complete myostatin gene sequence of *Bos taurus* (GenBank Accession No. AF320998.1) and *Bos indicus* (Accession No. AY794986.1) (NCBI, 2023).

RESULTS

PCR Amplification

The PCR amplification successfully generated targeted products, specifically a region s panning exons 3, with a total sequence length of 660 bp from the ATG start codon to the TGA stop codon. Figure 1 displays the outcome of the PCR amplification, showing PCR products of 660 bp. The primer designed for the myostatin gene was specific, resulting in a single DNA band observed in all the samples.

Myostatin Gene Mutations

In this study, Sanger sequencing analysis (Figures 2, 3, and 4) was employed to identify the specific mutations (deletions) within the nucleotide sequence of the myostatin gene among the Belgian Blue crossbred offspring.

The objective was to further confirm the successful introduction of the double-muscling trait to Malaysia's local beef cattle herd. However, it should be noted that not all samples selected for analysis exhibited the same deletion pattern. The results presented in Table 2, which includes 28 Belgian Blue crossbred offspring, revealed a variety of mutations (deletions).

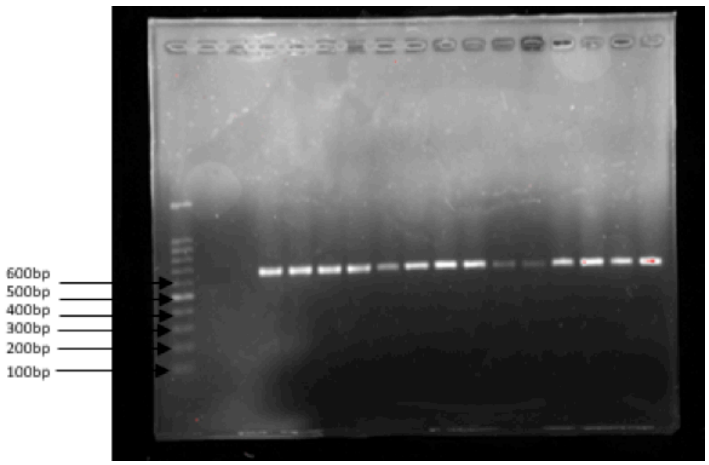

Figure 1. The PCR bands of a single amplified myostatin gene at 660-bp length were measured using a 100-bp ladder.

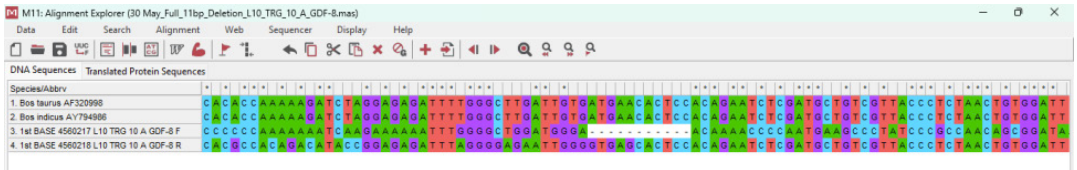

Figure 2. The 11-bp deletion of the nucleotides at the nt823-nt833 of the Belgian Blue crossbred offspring myostatin gene sequence compared to the normal myostatin gene sequence of Bos taurus and Bos indicus.

Figure 3. The 10-bp deletion of the nucleotides at the nt823-nt832 of the Belgian Blue crossbred offspring myostatin gene sequence compared to the normal myostatin gene sequence of *Bos taurus* and *Bos indicus*.

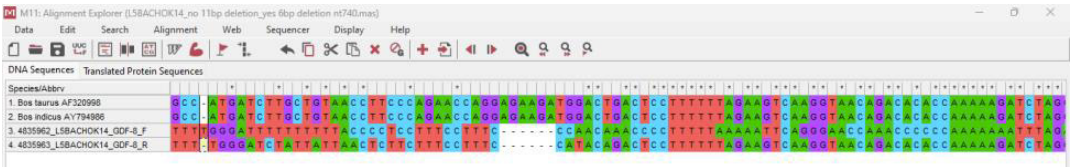

Figure 4. The 6-bp deletion of the nucleotides at the nt740-nt745 of the Belgian Blue crossbred offspring myostatin gene sequence compared to the normal myostatin gene sequence of *Bos taurus* and *Bos indicus*.

Table 2. The sequencing results of the Belgian Blue crossbreds sampled in the study born and raised in Malaysian smallholder farms in comparison with other related myostatin gene mutation studies.

No.	ID	Sex	Deletion (bp)	Nucleotide location (nt)	Deleted sequence	Related findings (References)
1.	L10TRG10A	Male	11	823	5′-ATGAACACTCC-3′	(Grobet *et al.*,1997; Jakaria *et al.*, 2021)
2.	255A	Male	11	823	5′-ATGAACACTCC-3′	(Grobet *et al.*, 1997; Jakaria *et al.*, 2021)
3.	258A	Male	11	822	5′-ATGAACACTCC-3′	(Grobet *et al.*, 1997; Jakaria *et al.*, 2021)
4.	L3BERA03B	Female	11	813	5′-CTTGATTGTGA-3′	-
5.	233A	Female	10	822	5′-TGAACACTCC-3′	(Rao *et al.*, 2016; Zhong *et al.*, 2016)
6.	L1TRG01	Male	10	823	5′-CACTCCACAG-3′	(Rao *et al.*, 2016; Zhong *et al.*, 2016)

7.	290AKD	Female	10	843	5′-TGCTGTCGTT-3′	(Rao *et al*., 2016; Zhong *et al*., 2016)
8.	L11TRG12	Male	10	666	5′-AACTTAGGCA-3′	(Rao *et al*., 2016; Zhong *et al*., 2016)
9.	L2KDH04	Male	10	665	5′-AACTTAGGCA-3′	(Rao *et al*., 2016; Zhong *et al*., 2016)
10.	292AKD	Male	6	784	5′-CCAAAA-3′	(Luo *et al*., 2014; Zhao *et al*., 2022)
11.	L5BACH14	Male	6	740	5′-AGAAGA-3′	(Luo *et al*., 2014; Zhao *et al*., 2022)
12.	L9KDH12	Male	6	740	5′-AGAAGA-3′	(Luo *et al*., 2014; Zhao *et al*., 2022)
13.	L12TRG13	Male	6	740	5′-AGAAGA-3′	(Luo *et al*., 2014; Zhao *et al*., 2022)
14.	BACHOK02	Female	6	739	5′-AGAAGA-3′	(Luo *et al*., 2014; Zhao *et al*., 2022)
15.	L3TRG03	Male	6	739	5′-AGAAGA-3′	(Luo *et al*., 2014; Zhao *et al*., 2022)
16.	289AKD	Female	6	736	5′-AGAAGA-3′	(Luo *et al*., 2014; Zhao *et al*., 2022)
17.	BACHOK04	Male	6	735	5′-AGAAGA-3′	(Luo et al., 2014; Zhao *et al*., 2022)
18.	253A	Male	6	729	5′-CCCAGA-3′	(Luo *et al*., 2014; Zhao *et al*., 2022)
19.	245A	Male	6	635	5′-AGACAG-3′	(Luo *et al*., 2014; Zhao *et al*., 2022)
20.	L5BACHOK16	Male	-	-	-	
21.	291AKD	Male	-	-	-	
22.	L2SELANGOR02	Male	-	-	-	
23.	L3SELANGOR04	Female	-	-	-	
24.	249A	Female	-	-	-	
25.	257A	Female	-	-	-	
26.	BACHOK01	Female	-	-	-	
27.	L4TRG4	Female	-	-	-	
28.	260A	Female	-	-	-	

DISCUSSION

The myostatin gene in cattle exhibits significant polymorphism. Most of the reported mutations in the coding regions (exons 1-3) of the myostatin gene are considered silent or result in non-synonymous alterations (Konovalova *et al*., 2021). The presence of myostatin gene mutations is not specific to a particular breed, as it has been observed in various cattle breeds, including *Bos taurus* (Bennett *et al*., 2019*), Bos indicus* (Grisolia *et al*., 2009), and *Bos javanicus* (Khasanah *et al*., 2016). This polymorphism can be harnessed through well-planned crossbreeding programs to enhance the genetic potential of beef cattle populations. The myostatin gene plays a crucial role in skeletal muscle growth, and mutations in this gene can negatively impact its function and skeletal muscle development (Bellinge *et al*., 2005) or growth and differentiation factor 8 (GDF8. However, with appropriate breeding strategies, this condition can be leveraged to yield economic benefits in farming operations. Charlier *et al*. (1995) conducted a study that mapped the mh locus to a position 3.1 cM from the microsatellite TGLA44 on the centromeric end of bovine chromosome 2. Subsequently, Kambadur *et al*. (1997) identified an 11-bp deletion of the nucleotides at the location of 821 in the open reading frame of the myostatin allele-specific to the Belgian Blue breed. This deletion resulted in the loss of three amino acids (275, 276, and 277), followed by a frameshift after amino acid 274. The frameshift led to a premature stop codon downstream, specifically at amino acid 287, resulting in truncation of the protein and preventing translation of a significant portion of the identical section lost in animals with the double-muscled phenotype (Tantia *et al*., 2006). Previous research by Grobet *et al*. (1997) demonstrated through a positional candidate approach and analysis of the bioactive carboxy-terminal domain of the protein that the mutated myostatin gene is indeed responsible for the double-muscled phenotype. These findings served as a valuable foundation for the current study, which focused on specific chromosomal regions (exons 3 of chromosome 2) to confirm the presence of similar mutations at the genotype level.

The results showed that mutations (deletions) occurred in the myostatin gene among the Belgian Blue crossbred offspring produced from crossbreeding between *Bos taurus* and *Bos indicus* breeds and raised under smallholder farm conditions in Malaysia. However, these mutations were found at different bp and nt. Three male Belgian Blue crossbreeds exhibited an 11-bp deletion at nt823, consistent with the findings reported by Grobet *et al*. (1997). While the 11-bp deletion at nt821 of exon 3 and its association with the Belgian Blue breed have been widely reported (Konovalova *et al*., 2021). McPherron and Lee (1997) reported a slightly different 11-bp deletion in the Belgian Blue breed, consisting of a deleted sequence (5'-TGAACACTCCA-3') at nt937-nt947. Jakaria *et al*. (2021) reported the same 11-bp deletion with the deleted sequence (5'-ATGAACACTCC-3') as reported by Grobet *et al*. (1997), which was found at nt818-nt828 of exon 3 among Belgian Blue and Peranakan Ongole crossbred cattle raised in Indonesia. These crossbred cattle also exhibited higher birth weights compared to purebred Peranakan Ongole cattle. It suggests that myostatin mutations may undergo genetic shifts, especially in crossbreeding involving two distant families, Bos taurus and Bos indicus. The 10-bp deletions discovered in myostatin gene during this investigation have not been reported in previous studies on the bovine myostatin gene. However, they have been observed in other species. For example, in gene-modified

porcine, the 10-bp deletion resulted in a double-muscled phenotype, with higher live weight and significantly larger longissimus muscle mass compared to wild-type piglets, as well as double the number of muscle fibres (Rao *et al*., 2016). In another study involving common carp (aquaculture fish), a 10-bp deletion led to significantly increased muscle cell growth in the mutated fish (Zhong *et al*., 2016). Five male and two female Belgian Blue crossbreeds in this study exhibited a 6-bp deletion with the deleted sequence (5'-AGAAGA-3') flanking nt735, nt736, nt739, and nt740 of the myostatin gene. This specific deletion has not been reported in any previous Belgian Blue breed studies. However, the 6-bp deletion of the myostatin gene has been found to have a significant effect. Luo *et al*. (2014) reported that genetically modified marker-gene-free cloned Chinese Yellow cattle with myostatin biallelic mutations, including a 6-bp deletion in one allele, exhibited hypertrophied quadriceps muscle fibres and the appearance of the double-muscled phenotype at one month of age. Furthermore, Zhao *et al*. (2022) conducted a study on growth traits and sperm proteomics analyses of myostatin gene-edited Chinese Yellow cattle and found that deletion of a 6-bp myostatin gene exhibited significant differences in live weight compared to non-gene-edited cattle, particularly at 24 months of age (593.59 kg for gene-edited, 485.73 kg for non-gene-edited). The other Belgian Blue crossbred offspring in this study that did not display any significant difference in myostatin gene mutations (deletions) were presumed to be influenced by the dominant factor of the normal-muscled Bos indicus genes inherited from the dam side.

The benefits of the mutated myostatin gene can be optimized while minimizing its potential drawbacks. It has been observed that the nt821del (11-bp) mutation is not exclusive to the Belgian Blue breed but is shared by several other breeds as well, indicating gene migration between populations (Grobet *et al*., 1998). Previous studies have suggested that the double-muscling phenotype is fully expressed when cattle have a homozygous recessive genotype for the mutated myostatin gene (mh/mh) (Kambadur *et al*., 1997). However, Casas *et al*. (1998) found that beef cattle inheriting a single copy of the mutant mh allele from a crossbred Belgian Blue showed increased longissimus muscle area. Agung and Said (2014) also confirmed this through gene sequencing analysis, revealing that the Belgian Blue crossbred F1 generation inherited the 11-bp deletion. PCR and sequencing analysis conducted by Grobet *et al*. (1998) demonstrated that the double-muscling phenotype in cattle is genetically heterogeneous, involving at least five different mutations in the bovine myostatin gene. Charlier *et al*. (1995) provided evidence of minor but significant muscle development superiority in heterozygous (nt821(del11)/+) normal cattle compared to homozygous wild-type (+/+) cattle. It is speculated that cattle with a single copy of an inactive myostatin gene exhibit increased muscle mass and reduced fat compared to regular cattle. Additionally, they are less likely to experience reproductive problems compared to purebred double-muscled cattle (Casas *et al*., 2000). Previous research by Widyas *et al*. (2018) supported these assumptions, demonstrating that calves with heterozygous mh alleles are slightly larger at birth but have comparable calving ease to calves without the mutated gene. Bittante *et al*. (2018) found that heifers born to Belgian Blue sires and Brown Swiss cows by artificial insemination exhibited a higher growth rate and carcass yield compared to heifers sired by another double-muscled

breed, namely Piedmontese. Furthermore, the South Devon breed has long been associated with double-muscling. Despite the relatively common occurrence of the deleted allele (0.4), breeders have generally selected for an intermediate phenotype, favouring good muscle conformation rather than double-muscled individuals (Smith *et al*., 2000).

Myostatin gene polymorphisms have been identified in Nellore cattle (*Bos indicus*) in Brazil, where sequencing of PCR products from multiple regions, including exon 1-3 and the untranslated region (UTR), revealed genetic variations (Grisolia *et al*., 2009). Jakaria *et al*. (2021) found that the first generation of Belgian Blue and Peranakan Ongole crossbred offspring exhibited high levels of polymorphism compared to purebred Peranakan Ongole cattle. High polymorphism indicates greater genetic diversity, which can contribute to the genetic advancement of selection strategies (Bunning *et al*., 2019). The suitability of incorporating Belgian Blue genetics in various locations depends on environmental factors, management practices, and the specific objectives of the breeding program. In certain tropical climates, the adoption of Belgian Blue genetics may require adjustments to cattle management methods to enhance livestock performance. Research conducted by the Brazilian Agricultural Research Corporation (EMBRAPA) showed that crossing Nellore cattle with Belgian Blue improved carcass traits. However, it also brought about an increase in the incidence of calving problems and dystocia, both of which could be mitigated through modifications in nutritional management (Grisolia *et al*., 2009). In a study conducted in Colombia, crossbreeding Brahman cattle with Belgian Blue resulted in improved growth rates, feed conversion efficiency, and carcass traits without affecting calving ease or dystocia rates (López-Paredes *et al*., 2017). This indicates that the successful adaptation of Belgian Blue genetics in tropical settings depends on factors such as the local cattle breed and existing management practices. In order to attain the most favourable results when incorporating Belgian Blue genetics into crossbreeding programs on a global scale and in tropical regions, it is required to make careful consideration of breed selection, management approaches, and genetic evaluation. Further studies are needed to fully understand the potential advantages and challenges associated with implementing such programs. In addition to management modifications, using Belgian Blue genetics in crossbreeding programs may require careful selection of breeding stock and genetic assessment of desired traits. Calving problems associated with double-muscling have led some breeding organizations to consider it as a genetic deficiency to be eliminated. However, in specific economic contexts, the benefits in terms of growth, carcass yield, and meat quality have proven to outweigh the drawbacks of dystocia. As a result, there has been a deliberate and consistent effort to select double-muscled cattle or incorporate their use into crossbreeding (Grobet *et al*., 1998). Therefore, the strategic breeding of double-muscled and normal-muscled beef cattle breeds in Malaysia presents to maximize growth performance without compromising reproductive or meat quality characteristics. This approach relies on the advantages of breed complementarity and heterosis (Bunning *et al*., 2019). There is a scarcity of data on myostatin mutations in *Bos indicus* in comparison to *Bos taurus*, which is necessary to investigate the possible application of the double-muscling trait in beef cattle specifically in tropical regions such as Malaysia. However, the process of selective

breeding often requires long-term phenotype screening and time-consuming of hybridization breeding to introduce pre-existing mutations in genetically complex domestic cattle. Therefore, rapidly, and easily implementing specific mutations in domestic beef cattle herds could be valuable potential solution for improving beef cattle breeds. The utilization of zinc-finger nuclease (ZFN) for gene targeting has shown significant effectiveness across multiple species. According to Luo *et al*. (2014), the research efforts to enhance the development of methods for fine-scale genetic modifications in domestic cattle have been reinvigorated. The potential to enhance income within the Malaysian beef cattle industry (comprises smallholder farmers who raise cattle in the backyard) lies in the introduction of the double-muscling trait to local beef herds. With limited herd space and scarce resources, producing heavier live weight in double-muscled crossbred cattle is expected to yield higher profits than normal-muscled local indigenous breeds like Kedah-Kelantan.

CONCLUSION

The molecular findings of the study confirmed the presence of the expected myostatin gene mutations (deletions) among the Belgian Blue crossbred offspring raised in a Malaysian smallholder farm setting. Therefore, incorporating of the double-muscling trait through the Belgian Blue breed in the crossbreeding program for beef cattle in Malaysia offers notable benefits. However, it should be noted that the detected myostatin gene mutations (deletions) originating from the crossbreeding program exhibited variability. Despite this variability, the introduction of the double-muscling trait enhances genetic diversity within the local beef cattle population and therefore yields to significant improvements in live weight. It is essential to carefully plan and implement crossbreeding strategies to develop the next generation of heterozygous double-muscled crossbreed beef cattle while maintaining the base population of local indigenous breeds to preserve tropical adaptability traits. Transitioning from traditional rearing of local indigenous normal-muscled breeds to double-muscled Belgian Blue crossbreeds requires an economic evaluation to aid smallholder farmers in making informed decisions for their farms. Evaluating the net benefits of rearing Belgian Blue crossbreeds, as well as considering the government-funded genetic interventions, is crucial for guiding decision-making processes.

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