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Research Article

Genotyping of Two Major Genes in Livestock by High Resolution Melt

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Abstract

Identification of genes affecting production is one of the important priorities in animal breeding strategies. The introgression of genes with a larger effect on quantitative traits into livestock can improve protein and economic resources. The present study aimed to evaluate high-resolution melting (HRM) analysis for genotyping bone morphogenetic protein receptor type 1B (Fecundity Booroola gene or FecB) in sheep and myostatin (MSTN) in cattle. This study was conducted on blood samples from 19 cattle and 170 sheep including: 19 control samples with approved FecB genotypes and 151 samples with unknown genotypes. After DNA extraction from blood and semen samples, a 176-bp fragment of the MSTN gene in cattle and a 140-bp fragment of the FecB gene in sheep were amplified. HRM was optimized and conducted in all samples then, the PCR-RFLP and sequencing methods were done to prove the HRM genotyping results. The results obtained using the HRM method matched those from sequencing and PCR-RFLP, confirming the effectiveness of HRM for genotyping and enabling rapid and accurate identification of individuals with FecB and MSTN mutations. Melting temperature range for MSTN genotypes was between 80.90 and 81.60 ºC and for FecB genotypes was between 79.6 and 80.50 ºC. Utilizing the HRM method for genotyping will provide researchers and commercial animal producers' access to a low cost, and easier method to genotype for any SNP detection. In this study, we successfully established a genotyping method for two loci in cattle and sheep economically important genes by high-resolution melting method. This method could be used in a large sample size with a high level of confidence.

***Keywords*:** High resolution melting, Genotyping, DNA marker, Economic trait, Double muscle, Fecundity

Introduction

It is predicted that global demand for animal feed increases by 70 % until 2050 (Gorgest et al, 2019). In recent years in our country due to the special animal husbandry system and food culture as well as increase in population, has paid much attention to animal breeding from a breeding perspective to increase productivity in animal protein production, major genes with a considerable impact on productivity must be introduced into livestock. Identification of genes affecting production is one of the most important priorities in animal breeding strategies (Mosher et al 2007). There are different types of DNA molecular markers, such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Single-Strand Conformation Polymorphism (SSCP), Tetra-ARMS and Microsatellite DNA (Nassiry et al, 2006, Nassiry et al, 2007, Aslaminejad et al, 2010, Tohidi et al, 2023). All these DNA-based markers contain specific advantages and have played significant roles in the evaluation of genetic diversity in farm animals. Advances in molecular genetics have introduced new generations of molecular markers for use in the genetic improvement of farm animals.

To meet this demand, modern animal breeding increasingly relies on precise molecular tools, with High-Resolution Melting (HRM) analysis emerging as one of the most efficient and accessible technologies. HRM is a PCR post-analysis method used for detecting genetic diversity in nucleic acid sequences. In this method, the samples are accurately classified based on melting point of PCR products. If a DNA fragment has a change in its nucleotides, its melting point will be different from the other samples (Javadmanesh et al., 2022). In the HRM method, the samples are classified in terms of melting curve. In this method, if a sequence has changes in its nucleotides, its melting curved is

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different from other samples; as a result, its graph is different from other samples. The HRM characterizes nucleic acid samples based on their disassociation behavior and detects small sequence differences in PCR-amplified sequences, just by direct melting. Samples are also discriminated according to their sequence length, GC content and strand complementarity. With the use of specific DNA dyes, high-end instrumentation and sophisticated analysis software, these differences are detected (Kim et al., 2023). HRM can detect the differences between SNP allele melting temperatures by using a fluorescent dye inserted into the duplex deoxyribonucleic acid (DNA) structure (Tong et al, 2012). HRM reactions are closed tube, which reduces the risk of contamination. In addition, HRM assays are non-destructive so that the actual sample used in mutation scanning can serve as sequencing template (Nikodem et al., 2021).

Several candidate genes known for milk and meat production, and other economic traits in domestic animals (Vanek et al, 2008). There have been constant trends for improvement in the quality of carcasses and more lean meat in different breeds of cattle since more lean meat is good for the meat industry and health (Vanek et al, 2008). Animals with double muscle phynotype can produce more meat with less fat while using fewer resources mostly due to a mutation in growth and differentiation factor-8 gene (Soleimani et al., 2019). In the 1970s, much research had been done on animals with double muscle that can be referred to as Piedmontese and Belgian Blue cows (Kambadur et al, 1997). Growth and differentiation factor-8, is a protein secreted by muscle and adipose tissues, also referred to as Myostatin (MSTN) (Riasi et al., 2022; Soleimani et al. 2019). MSTN is one of the most powerful negative regulators of muscle growth and differentiation. Regulation of MSTN gene expression can accelerate muscle growth and animal production without known side effects. Thus, an inhibitor of MSTN may be therapeutically useful as an anabolic agent for the muscle tissue (Riasi et al, 2024). The MSTN gene is located on bovine chromosome 2 and consists of three exons and two introns (Dybus et al, 2013). A mutation in the MSTN gene has a significant effect on the increase in body composition and improvement of carcass traits such as lean meat percentage, fat content, etc. Belgian Blue breed of beef cattle is known as double-muscled cattle. Further analysis indicated that a 11-bp deletion in the exon III of MSTN gene in Belgian Blue cattle could cause the double-muscled trait, a phenotype characterized by a significant increase in muscle mass (Chelh et al, 2010). Belgian Blue beef cattle are popular worldwide due to the taste, marbling, and tenderness of their meat. Crossing of a Belgian Blue with different breeds such as Angus, Jersey, Nelore, Simental, Montbeliard, and Brahman showed difficulties in calving due to the size of the calf and the small size of the cervix, therefore caesareans and death were increased (Muller et al., 2004). Insemination Holstein cow with Belgian Blue semen improves carcass performance, improved feed efficiency, good taste, tenderness and higher marbling and no negative effect in parturition (Deng et al, 2012). As a result, crossing this breed with some dairy individuals could be beneficial. Recently, in Iran, there have been some examples of hybridization between Belgian blue and Holstein cattle.

The most important factor of profitability in sheep breeding is meat production, which can be achieved through the highest rate of reproduction- a polygenic trait (Gootwine, 2020). Fertility and reproductive efficiency are two key factors affecting the economy of the sheep breeding industry (Wang et al 2000). Relevant studies have shown that major fecundity genes can significantly increase the number of multiple births, thereby improving the reproductive efficiency of sheep and economic conditions of sheep farmers (Yao3-Jing et al., 2011). FecB (Booroola fecundity) was originally found in Australian Merino sheep named Booroola during the 1980s (Javadmanesh et al, 2004), and it has been proven that can cause a great effect on ovulation rate and reproductive function of sheep (Rashidian and Javadmanesh, 2023). The litter size is positively correlated with the copy number of the FecB mutation. Research has shown that heterozygote (B+) resulted in 93% more lambs than the wild type (++) in sheep and that the B+ genotype also had a 65.6% higher litter size than the ++ genotype (Mishra et al., 2009). Further studies have shown that sheep with the BB genotype or B+ genotype had significantly higher litter size than those with the ++ genotype (Fogarty et al, 2009). An increased production rate between 60 to 70% also observed in different countries, including India (Kumar et al, 2021). Recently, the frequency of the FecB mutant allele in sheep has been increasing rapidly in Iran (Rashidian and Javadmanesh, 2023) meanwhile, the frequency of the MSTN mutant allele in cattle is increasing slowly.

In this study, HRM was used for genotyping MSTN and FecB genes, in a number of sheep and cattle blood samples. Accuracy of these findings was assessed with PCR-RFLP and sequencing methods.

Materials and Methods

Collection of samples

This study was conducted on blood samples from 170 sheep, including three control groups of approved FecB genotypes and one test group with an unknown FecB genotype, as described below:

Control 1: 13 homozygotes samples without FecB mutation from purebred Iranian breeds collected from sheep breeding stations (5 Kurdi from Kurdi Breeding station located in Shirvan, North Khorasan Province, 5 Karakul and 3 IranBlack samples from Abbas Abad Breeding station, Mashhad, Khorasan Razavi Province).

Control 2: Five heterozygote samples of Kurdi-Afshari-Booroola hybrids from Kurdi Breeding station located in Shirvan, Khorasan.

Control 3: One homozygotes sample with FecB mutation from Kavosh Gene Sarvdasht laboratory (Mashhad, Khorasan Razavi Province).

Test group: 151 blood samples with unknown genotypes from different herds located in Khorasan Razavi Province, Iran.

Also 17 samples from cattle including nine samples of Belgian Blue-Holstein hybrids with one copy of MSTN mutation, seven samples from pure-bred Holstein cattle with no MSTN mutation and one frozen semen sample of the pure-breed Belgian Blue with two copies on MSTN mutation. All blood samples were collected from Behparvaran Holstein Ray dairy farm, Tehran. Since the frequency of MSTN mutation in Iranian cattle is very rare and only one genotype was available, there was no test group for MSTN genotyping.

Approximately two ml of blood was collected from of each animal in EDTA-containing vacutainer tubes.

Blood DNA isolation

Total genomic DNA was extracted from whole blood using the NEXprep™ Blood DNA Mini Kit (NEX Diagnostics, South Korea) and the DNA was stored at −20°C. The integrity of extracted DNA samples was assessed by agarose gel electrophoresis. The quantity and purity of DNA were evaluated by Epoch Microplate Reader instrument (BioTek Instruments, USA).

DNA extraction from frozen sperm

DNA from the semen sample was extracted using a modified protocol (Miller et al., 1988). The sperm pellet was lysed with 450 µl of prewarmed (60°C) digestion buffer (10 mM Tris pH 8·0, 10mM EDTA pH 8·0, 1% SDS, 100 mM NaCl) followed by the addition of 50 µl of 0·5M freshly made dithiothreitol, and 20 µl of Proteinase K solution (20 mg/ml). The solution was incubated overnight at 60°C and 20 µl of fresh Proteinase K solution (20 mg/ml) was added and incubated at 55°C for another 2 h. Following incubation, 170 µl of saturated NaCl solution was added and the mixture was shaken vigorously for 1 min. The solution was then centrifuged for 10 min at 15 000 g. The supernatant was added to 1.3 ml of ethanol and mixed gently to allow the DNA to precipitate. The precipitated DNA was centrifuged at 15000 g for 10 min. Then the supernatant was removed and washed the pellet with 300 µl of Ethanol 70%. Another round of centrifuge at 15000g for 2 min was conducted. The pellet was dried in 65 °C for 10 min and finally 100 µl of TE buffer was added to dissolve DNA.

Primer design and PCR amplification

DNA sequences of MSTN and FecB genes with accession numbers of NC_037329.1 and NC_040257.1 were obtained from NCBI database (www.ncbi.nlm.nih.gov). For each gene, one pair of primers was designed using Primer Premier 5 software (www.premierbiosoft.com) (Table 1). Primer pairs were designed to amplify a short fragment suitable for HRM, PCR-RFLP and PCR-SSCP methods to further confirm the genotyping results. A fragment of exon III from bovine MSTN gene with length of 176 bp and exon VIII of ovine FecB gene with length of 140 bp were amplified using specific primers. The PCR program for amplification of both genes was performed with the following condition: one cycle at 95 °C for 10 min followed by 38 cycles of 94 °C for 30 sec, 58 °C for 20 sec, 72 °C for 20 sec and one cycle of 72°C for 5 min as final extension. Reactions were in duplicates and each reaction contained 7.5 µl Red MasterMix (2X) (Ampliqon, Denmark), 2 µl template DNA (equal to 50-100 ng), 2.5 µl ddH_2O, 3 µl of primer mix (7.5 pM of each strand) in a total reaction volume of 15 µl.

Genotyping of mutations in bovine MSTN and ovine FecB by HRM

All reactions were duplicated in a total volume of 15 µl containing: 3µl of 5x HOT FIREPol EvaGreen HRM Mix (no ROX) (Solis Biodyne, Estonia), 2 µl of DNA, 2 µl of primer mix and 8 µl of ddH_2O. Samples were assayed using the LightCycler® 96 real-time PCR System (Roche Life Science, Switzerland) with the following thermal cycling and melting conditions used for both genes: 95°C for 15 min; 40 cycles of 95°C for 15S, 60°C for 20S and 72°C for 30S, followed by a high-resolution melt of 70°-90°C (0.05 °C/S, 20 reading/°C).

Table 1. Primers designed for MSTN and FecB gene amplification

Gene symbol	Annealing temperature (°C)	Primer sequences	Fragment length (Base pair)	Fragment GC (%)	Accession number
MSTN	58	F-CCTTGAGGTAGGAGTGTTTTGG R-CACAGTTAGAGGGTAACGACAGCAT	176	49	NC_037329
FecB	58	F-CAAACTTCCCATAGCGACCT R-GTCCAGAGGACGATAGCAAAGC	140	52	NC_040257

Data were acquired and analyzed using the accompanying software version 1.1 provided with the LightCycler® 96 System (Roche Life Science, Switzerland). Normalized and temperature-adjusted melting curves of test samples and controls were compared (Javadmanesh et al., 2022).

PCR-RFLP and SSCP analysis

To validate the sensitivity and specificity of HRM results in both genes, PCR-RFLP and PCR-SSCP used for FecB and MSTN genes, respectfully. Primers used in HRM reaction were utilized in both PCR-RFLP and SSCP (Single stranded conformation polymorphism) reactions to confirm the genotypes of FecB and MSTN genes (Table 1). PCR program and reaction conditions were described already in Primer design and PCR amplification section. Digestion reaction mixtures (15 µl) contained 5 µl PCR product (FecB gene), 1.5 µl digestion Buffer R (10X), 0.5µl *Ava*II (10U/ µl) (Thermofisher Scientific, USA), and 8 µl ddH_2O. After digestion, we expect FecB mutation yield two 110 and 30 bp fragments (B/B), non-carrier products remain uncut at 140 bp (+/+). Heterozygotes should produce three fragments of 140, 110 and 30 bp (B/+). Digestion products were screened by 2.5% agarose gel electrophoresis. The genetic variability in exon III of MSTN gene in cow was assessed on an 8% polyacrylamide gel electrophoresis. Two µl of MSTN PCR product mixed with 8µl SSCP dyes (10 µl bromophenol
10% + 2 µl of EDTA at 0.5 M + 190 µl glycerol + 800 µl of formamide) for 5 min at 95°C. Then the samples were placed on ice for 10 min to prevent the reconnection of the complementary strands to each other. The samples were electrophoresed in a cold water-circulated 8% acrylamide gel with a voltage of 320 V for 150 min (Nassiry et al, 2010). The gel was visualized using silver staining method.

Sequencing

As golden standard of genotyping, three individuals from each genotype were selected to confirm the mutation with Sanger sequencing. The PCR products then purified by ethanol precipitation method prior to Sanger sequencing. 25µL of products PCR was mixed with 75µL of 96% ethanol. Stored for 15 min at -20°C, then centrifuged at 10000 rpm for 5 min. Supernatant was removed and pellet was dried. 200 µL of 70% ethanol was added, and after shaking vigorously, stored for 8 min at -20°C. Then centrifuged at 14000 rpm for 5 min and supernatant was removed. The pellet was incubated for 4 min at temperature of 65°C to dry and finally 30µL of deionized water was added to dissolve the purified PCR products. Samples were sequenced by standard Sanger sequencing method. The sequencing results were evaluated by BLAST (Jian et al, 2006).

Results

The results of spectrophotometry and electrophoresis of the extracted DNA showed suitable quality. The fragments of exon III of the MSTN gene with a length of 176 and exon XIII of the FecB gene with a length of 140 bp were amplified specifically. The evaluation of sequencing results proved that mutant homozygote samples of FecB gene had two-point mutations (A to G), while the wild-type homozygotes had no mutations. In addition, determination of the nucleotide sequence in the MSTN gene showed that mutant homozygotes had an 11 bp-deletion in exon VIII region, which caused a frameshift mutation in MSTN gene of Belgian Blue cattle.

PCR-RFLP genotyping results for the FecB gene were 100 % in accordance with HRM genotypes in all 151 unknown genotype samples (Figure 1). The frequencies of wild-type genotype, heterozygotes, and mutant homozygotes were 0.45, 0.53, and 0.02 respectively. Since these samples were taken from several different herds, calculating Hardy-Weinburg equilibrium was not meaningful. In addition, PCR-SSCP genotyping showed three different patterns and confirmed the results of HRM genotyping for

the MSTN gene (Figure 2). HRM is done on control samples with known genotypes to determine melt curve profile and difference graph (Figures 3 and 4). The findings confirmed that the HRM analysis results are aligned with the sequencing of MSTN and FecB genes in all control individuals.

In MSTN gene, different samples of one genotype showed very similar melting curve patterns and could be grouped with confidence of 95% for each genotype (Figure 3). MSTN mutant homozygote samples showed a melting temperature of approximately 80.90 ºC, while heterozygote individuals and wild-type homozygotes showed melting temperatures of 81.30 ºC and 81.60 ºC, respectively. In the wild-type genotype, the length of exon is longer than other two genotypes due to the absence of mutation in exon III. As a result, the melting point temperature was higher than two other genotypes.

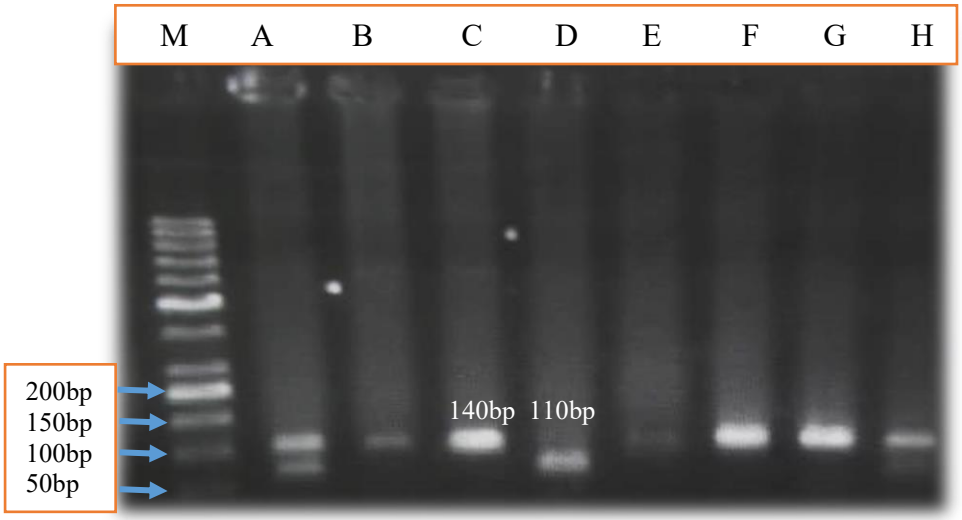

Figure 1. 2.5% agarose gel electrophoresis of AvaII digestion of a 140-bp fragment from ovine FecB gene. M: molecular size marker, A and H: heterozygous, D: mutant homozygous, B, C, E, F, G: wild type.

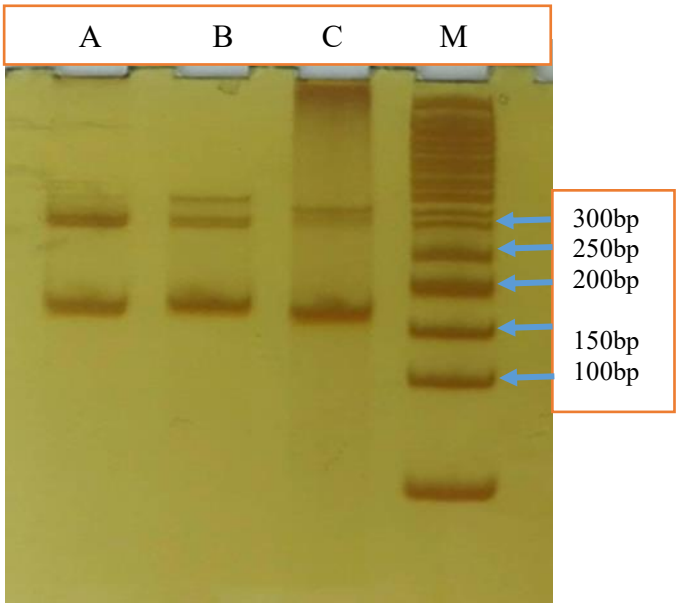

Figure 2. Electrophoresis of the 171-bp fragment from bovie MSTN gene by Single starnded conformation polymorphism on an 8% polyacrylamide gel. A (Belgian blue), B (Holstein), C (Belgian blue-Holstein hybrid), M: molecular size marker.

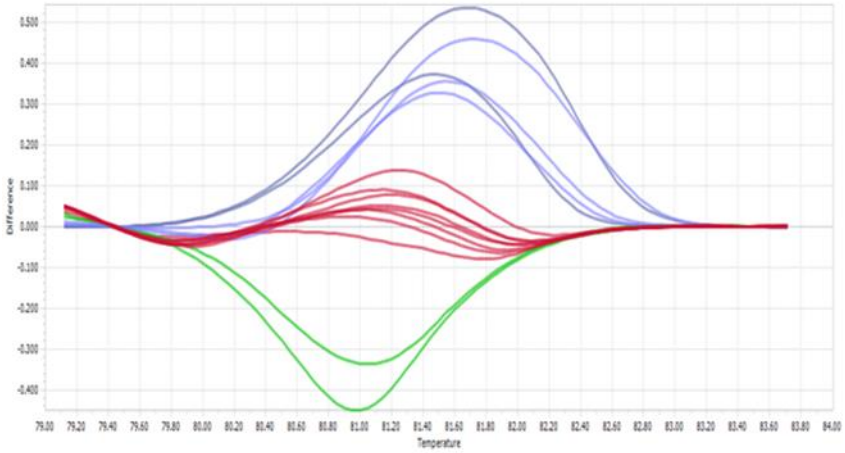

Figure 3. High Resolution Melting difference graph for MSTN (mutant homozygous (green), heterozygous (red) and wild-type (blue))

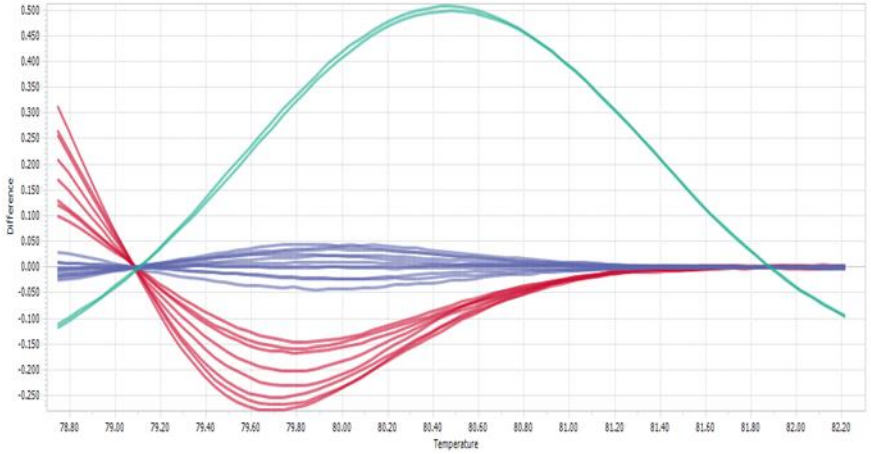

Figure 4. High Resolution Melting difference graph for FecB (Booroola homozygous (green), heterozygous (blue) and wild-type (red))

Discussion

The primary objective of this study was to establish and validate a High-Resolution Melting (HRM) protocol for the rapid, accurate, and cost-effective genotyping of two economically significant genes in livestock, including the Myostatin gene (MSTN) in cattle and the Fecundity Booroola gene (FecB) in sheep. The successful development and validation of these closed-tube assays provide a highly efficient alternative to traditional methods, addressing the critical need for high-throughput genotyping in modern animal breeding strategies (Gorgest et al, 2019). The central finding of this research is the 100% concordance between the HRM results and those obtained from the gold-standard Sanger sequencing, as well as the established conventional methods (PCR-RFLP for FecB and PCR-SSCP for MSTN). This complete agreement rigorously validates the HRM assays for both loci. HRM analysis effectively and precisely distinguished all three possible genotypes wild type, heterozygous, and mutant homozygous for both the FecB point mutation and the MSTN 11-bp deletion. This high level of accuracy, demonstrated across 170 sheep and 17 cattle samples, including the 151 unknown FecB samples, strongly advocates for the widespread adoption of this technique. Compared to PCR-RFLP and PCR-SSCP, which are labor-intensive, time-consuming, and carry a risk of gel-based false positives or band resolution issues, the HRM method offers significant operational advantages. It is a closed-tube system, minimizing the risk of contamination and post-PCR processing (Nikodem et al., 2021). Furthermore, while Sanger sequencing remains the definitive validation tool, its high cost and low throughput make it impractical for routine commercial screening. The established HRM protocol eliminates the need for expensive post-PCR steps, positioning it as an invaluable tool for researchers and commercial producers seeking to screen large cohorts of animals rapidly and

economically (Tong et al, 2012). The FecB mutation in the BMPR1B gene is a major determinant of prolificacy in sheep, directly enhancing ovulation rate and litter size key drivers of productivity and profitability in sheep farming (Gootwine, 2020; Fogarty et al, 2009). The ability to accurately and quickly genotype this mutation is essential for screening the introgression of the desirable B allele into local Iranian breeds. In the 151 unknown samples analyzed, the detected genotype frequencies (Wild Type +/+: 0.45, Heterozygotes B/+: 0.53, and Mutant Homozygous B/B: 0.02) suggest an active and relatively successful introgression program. The prevalence of the heterozygous B/+ genotype (53%) indicates that breeders might have intentionally maintained the B allele, which provides a balance between increased lambing rates and potential maternal complications sometimes associated with the homozygous B/B state. This high frequency of mutant FecB allele in native sheep breeds is an indicator of introgression of FecB mutation into these breeds since this mutant allele was not present in any of native sheep breeds around two decades ago in Iran (Javadmanesh et al., 2004; Qanbari et al., 2009).

The successful application of HRM here enables breeders to effectively monitor their flock genetics, ensuring the most advantageous animals are selected for breeding a process that must be continuously optimized given the recent increase in FecB allele frequency in the Iranian sheep population (Rashidian & Javadmanesh, 2023). This simple, validated HRM test is therefore a powerful and practical means to accelerate genetic gain in fertility traits, contributing directly to increased lamb output and farm profitability. Similarly, the MSTN gene is a crucial target for meat production. It acts as a negative regulator of muscle growth, and the 11-bp deletion in exon III disrupts this function, leading to the double-muscling phenotype characterized by pronounced hypermuscularity (Chelh et al, 2010). This translates into superior, economically vital carcass traits, including higher lean meat yield and improved feed conversion efficiency (Vanek et al, 2008). The HRM difference graph for MSTN clearly separates the three genotypes, with the wild-type exhibiting a higher melting temperature (81.60ºC) compared to the heterozygous (81.30ºC) and homozygous mutant (80.90ºC) samples. This distinct temperature profile is directly attributable to the change in fragment length caused by the 11-bp deletion, which weakens the duplex and lowers the melting point. This clear separation with a high confidence level (95%) highlights the robustness of the assay for quality control. The genotyping of crossbred cattle (Belgian Blue-Holstein hybrids) is of particular importance in this study. While the double-muscling trait is desirable for enhanced meat quality and yield, pure Belgian Blue genetics can lead to calving difficulties. Crossbreeding with dairy cows like Holstein is a practical strategy to introgress the beneficial muscle hypertrophy trait while mitigating dystocia risk (Deng et al, 2012; Muller et al., 2004). Some studies were performed on different regions of the MSTN gene in various animals using HRM method and results indicated that accuracy is very high. A region in 5'UTR of MSTN gene in rabbit was genotyped by HRM method and three genotypes identified as TT, TC, and CC (Peng et al., 2013). Another species which determination of MSTN genotypes was conducted using HRM is goat. It has been reported only two AA and BB genotypes (Ahmadi et al., 2024). MSTN genotyping in horses reported by Serpa et al (2017), similar to the current research, indicated that HRM analysis may be a faster and more economical alternative than PCR methods for genotyping (Serpa et al., 2017).

The HRM assay provides the practical tool needed to manage these critical breeding decisions. By rapidly identifying heterozygous (N/Del) or homozygous mutant (Del/Del) individuals, producers can precisely select for optimal levels of muscle development while controlling for calving ease in their herds. The successful implementation of HRM for both a Single Nucleotide Polymorphism (SNP) (FecB) and a small InDel (MSTN) demonstrates the versatility of this technology. This success paves the way for the rapid development of similar assays for other major genes affecting production traits in livestock. Genes controlling milk production, disease resistance, or other fitness traits could be efficiently added to a diagnostic panel using the same instrumentation and general protocol. The establishment of this method aligns with the broader global trend toward low-cost, decentralized molecular diagnostics. By providing a highly effective alternative to sequencing, this HRM protocol allows regional laboratories and breeding centers to perform large-scale genetic screening without reliance on specialized, expensive facilities, thereby democratizing access to modern livestock genomics. The ability to genotype economically important traits like fertility and muscle development quickly and with high confidence is crucial for ensuring sustainable and profitable animal production in the future.

Conclusion

High-resolution melting is inexpensive, flexible, and only mildly constrained by primer design. The ability of HRM to rapidly and accurately screen large sample sizes with high confidence makes it an invaluable tool for effectively monitoring and accelerating this introgression process, which is crucial for increasing productivity in animal production.

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Conflict of interests
None.

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