Identification of Myostatin Gene Polymorphism Using PCR-RFLP for Improving Carcass Meat Evaluation of Teleorman Black Head Lambs

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Abstract

The objective of this study was to identify Myostatin *(MSTN*) gene polymorphism using *PCR RFLP* technique in order to improve sheep meat evaluation. There are data which show that *MSTN* is an inhibitor of skeletal muscle growth and a mutation in gene leads to increased muscle mass. Therefore, it is considered as an appropriate candidate gene for carcass meat quality. Blood samples were collected from 105 Teleorman Black Head (TBH) lambs and *DNA* was extracted using, Wizard Genomic DNA Purification Kit. Polymorphism of *MSTN* was determined by *PCR* amplification followed by *RFLP* method using restriction enzyme *Hae III*. Based on results, two genotypes *mm* and *Mm* were identified. It was identified homozygous genotype *mm* which not carry this mutation with 16.67% and heterozygous genotype *Mm* with 83.33%. *M* allele frequency was 42% and for allele *m* was 58%. Observed and expected values of *MSTN* genotypes were found in Hardy Weinberg disequilibrium, after $\chi 2$ test was calculated. *MM* individuals who carry this mutation were not identified in the analysed herd. This may be due to an empirical selection of this breed leading to absence of this genotype. That is why further investigation on a high number of animals are necessary to discover this mutation correlated with meat quality in TBH sheep.

Keywords: myostatin, carcass, sheep, meat, evaluation, quality, polymorphism

1. Introduction

In our country investigations to identify the molecular markers and gene polymorphisms in sheep and goats were made by Balteanu et al., 2010, 2011 [1, 2] and Lazar et al.2010, [3] who studied the protein polymorphism of goat milk and its influence on Carpathian goat breed and F1 hybrids with Saanen. Myostatin gene was found in the literature either nominated *GDF8* or *MSTN*. Studies on gene expression and its influence on local sheep breeds for meat quality were not yet conducted in our country. That is why the aim of this study was to characterize the genotypic expression of Myostatin in Romanian Teleorman Black Head sheep breed. Myostatin recognized as growth and differentiation factor 8 GDF was physically genotyped on goat chromosome position 2q11q12 [4, 5]. It is considered to be an important candidate gene for growth and development of the animals due to its key role in muscle development and its potential applications in goats breeding [6, 7]. Gene myostatin is considered a candidate gene important since it influences growth and development and has been studied in ruminants, but also to non-ruminants, distinguished by variations bases to amino acids, which are expressed and recognized as double muscles development in cattle [8]. Point mutations in pig *MSTN* gene influenced and improved the average daily gain [9]. In hens Gu et al., 2002, [10] found five *SNPs* in the region of 5'...3'. Those

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protected polymorphic sites were strongly associated with production traits.

2. Materials and methods

Animals. The study was conducted on 105 Teleorman Black Head (TBH) lambs aged 3.5 months, in a farm from Teleorman County (Romania). *PCR-RFLP method* was used to determine polymorphism of *MSTN* gene. Blood samples were extracted from 105 lambs and the PCR products were digested with *Hae III* enzyme. PCR analyses were done using primers for specific amplification of *MSTN* gene:

Forward: CCGGAGAGACTTTGGGCTA
Revers: TCATGAGCACCCACAGCGC

PCR reaction was run in a total volume of 25 µl using 75 ng of extracted DNA with PCR mix of 7.7 µl: 4µl PCR buffer, 2 µl $MgCl_2$ (2.5mM), 0.4 µl dNTP (0.2mM), 0.6 µl forward and revers primers (0.3µM for each primer), and 0.1 µl (1.25U) Go Taq DNA polymerase.

PCR program: 2 minutes denaturation at 95^{O}C, 40 cycles with 1 minute denaturation 95^{O}C, 30 seconds elongation at 58^{O}C, 1 minute 72^{O}C extension, and final extension 10 minutes at 72^{O}C and then decreasing at 4^{O}C for being kept on conservation until is used in *RFLP* reaction. *MSTN* gene amplicon of 337 bp was visualized on 2 % agarose gel, DNA ladder was 100bp.

RFLP program for MSTN identification used was: 180 minutes at 37 ^{O}C using a *RFLP mixture* with a total volume of 5 µl: 2 µl Buffer C, 0.5 µl BSA, 0.5 µl *Hae III*, and 2 µl ultrapure water, total volume of the reaction: 20 µl with 15 µl of *MSTN* amplicon and 5 µl *RFLP* mixture. *RFLP* products after digestion was visualized on 4 % agarose gel.

Individuals homozygous were identified with restriction enzyme *Hae III* which cuts the amplicon in two places obtaining genotype *mm* with three migration bands at 131, 123 and 83 bp. Restriction enzyme doesn't cut the amplicon of the homozygous individuals for the genotype *MM*, and there is no restriction site, so amplified DNA fragment migration can be visualized in one band of 337 bp.

3. Results and discussion

In his study Soufy et al., 2009 obtained tree genotypes of *MSTN:* 2% *MM*, 1.33% *Mm* and 96.7% *mm*, different by the present study with the mutational genotype of myostatin *MM*. Individuals heterozygous with *Mm* have four DNA fragments that migrates, because the restriction enzyme cuts the amplicon in three sites and the size of each fragment are 337, 131, 123 and 83 bp. All these are illustrated in Table 1 and Figure 1 [11].

Table 1. Restriction fragments of *MSTN* gene in homozygous and heterozygous individuals (Soufy et al., 2009)

Item		Restriction fragments of MSTN gene (pb)		
Allele M no situs		337	-	-
Allele m 2 situs		131	123	83
MM	337	-	-	-
Mm	337	131	123	83
mm	131	123	83	-

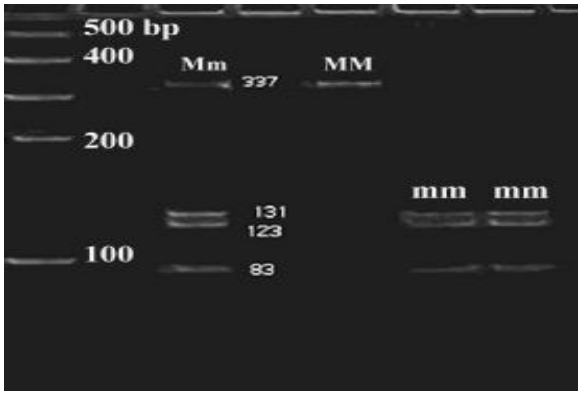

Figure 1. Genotypes differentiation of *MSTN mutation (Soufy et al., 2009)*

In this study, from the resulting genomic DNA the next step in PCR amplification of *MSTN gene* was the visualization of DNA fragment without restriction (Figure 2). Figure 3 and Figure 4 show the restricted DNA samples and each sample fitted according to the individual's genetic type (homozygous *mm*, *MM* or heterozygous *Mm*) of the myostatin gene.

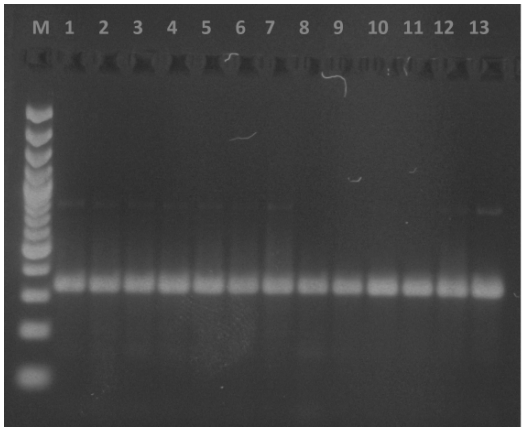

Figure 2. PCR amplicon of MSTN of 337 bp in Teleorman Black Head lambs 2% agarose gel, ladder 100 bp

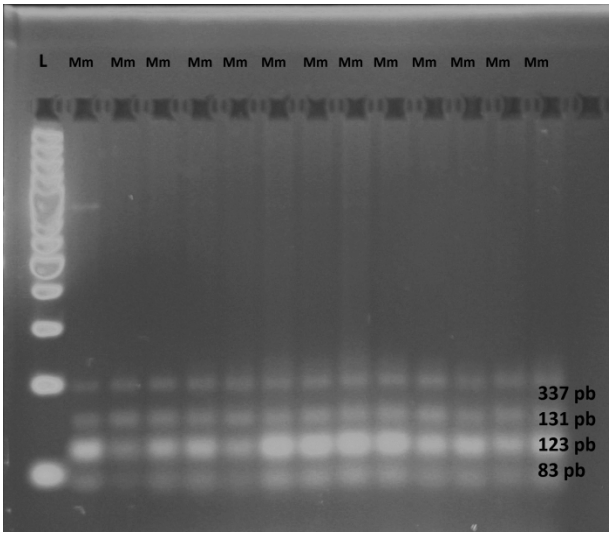

Figure 3. DNA restriction fragments with heterozygous individuals genotyped *Mm* in Teleorman Black Head lambs, 2% agarose gel, ladder 100 bp

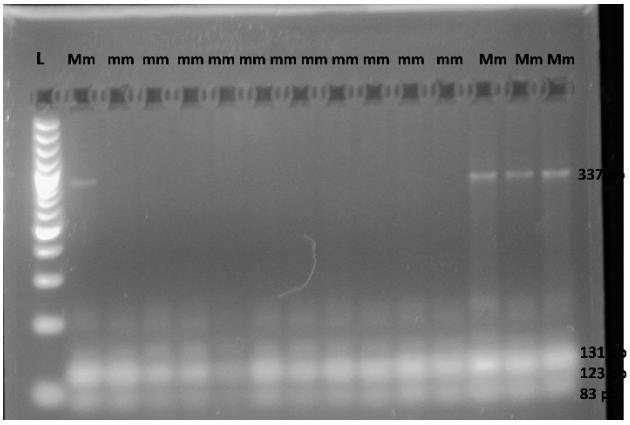

Figure 4. DNA restriction fragments with heterozygous individuals genotyped *Mm* and *mm* in Teleorman Black Head lambs, 2% agarose gel, ladder 100 bp

Based on results, were identified two genotypes of myostatin: *mm* and *Mm*, 16.67 % were *mm* and 83.33% which did not carried this mutation. We did not identified individuals homozygous with the mutation *MM*.

Gene frequency was 42 % for *M*, and 58 % for *m* (table 2). The expected values and the observed genotypes of *MSTN* were found in Hardy Weinberg disequilibrium after $\chi 2$ calculated test. This might be caused by an empirical work selection so far of this sheep breed of TBH detrimental to heterozygous genotype *MM*. In the present study we did not identified homozygous individuals with myostatin mutation *MM* and we found only homozygous and heterozygous individuals for genotypes *Mm* and *mm* (table 3, Figure 5). In his study Dehnavi et al., 2012, [12] had identified *MSTN* genotypes and their association with live weight in Zel sheep breed, a good method to find alleles affecting the quality and quantity of meat. Statistical analysis showed that *MSTN* locus had no significant effect on weight at one year of age. Using method *PCR-SSCP* the same author found the exons 1 and 2 with tree genotypes *AA*, *AB* and *BB* in *MSTN* gene of 73.5 %, 4 % and 22.5 %. The frequency of allele *A* and *B* of *MSTN* was 75.5% and 24.5% respectively. Similar results were obtained from Iranian Baluchi sheep [13, 14]. This inconsistency can be described by differences between breeds, the analysed herd, by the effective size of the animal herd, environmental factors, mating strategies, the effect due to the geographic distribution and genetic variants frequency. Statistical analysis showed that *MSTN* locus had not a significant effect on yearling weight. However, Masoudi et al., 2005 [14] observed a significant effect of different genotypes on weight at birth, but not on weight from weaning to the six months age. Ansary et al., 2008, [13] observed a significant effect of different genotypes on average daily gain from birth to 3 months age. Dehnavi et al., 2012 [12] studied myostatin specifically exon 3 by the molecular biology method of *PCR-RFLP* in Zel sheep breed and observed that analyzed individuals show no genetic polymorphism because after enzyme restriction only three fragments of 131, 123 and 83 bp were identified and all samples were genotyped *mm* compared to our study where we observed two genotypes of this gene *mm* and *Mm*, respectively. Zel sheep population analysed by

Dehnavi et al., 2012 [12] observed an HW disequilibrium, after $\chi 2$ test was calculated and this confirms that the factors who leads particularly to disequilibrium had been selected and this may affect the genetic structure of the population. There was a low degree of variability in *MSTN* gene, and this degree can be explained by the breed preservation and selection strategies that had been developed. Due to the small size of the analysed population, inbreeding was high and as a consequence, heterozygosity and genetic variability was low. Sorour et al. 2014 [15] identified specific DNA fragment with 337 bp exon 3 in Mehraban sheep breed using the same method of investigation as in the present study. Similarly, these authors obtained two genotypes *Mm* and *mm*, homozygous genotype *mm* recording a greater frequency (94.7%) and a lower frequency of heterozygous genotype *Mm* of 5.3% compared to TBH sheep frequency of 83.33% and 16.67%. In Mehraban sheep *m* allele frequency of *MSTN gene* was of 0.974, higher than TBH sheep which registered 0.580. But *M* allele frequency was only 0.026 in Mehraban sheep compared to TBH sheep which recorded a higher frequency of 0.420. In Mehraban sheep population after $\chi 2$ test was calculated. Hardy Weinberg disequilibrium was observed due to destructive factors such as migration and selection but also to the size of the analysed population. Svetlana Georgieva et al., 2015 [16] also studied in Bulgarian sheep milk, myostatin by *PCR* amplification followed by *RFLP* with *Hae III* as in the present study. She identified only three restriction fragments of 131, 123 and 83 bp in all analysed individuals, who designates a genetically monomorphic population with genotype *mm*. Ahan Azari et al., 2012, [17] also studied genes correlated to sheep meat production, gene related with carcass meat quality and quantity, in exon 3 of myostatin gene, in Dalagh sheep. In this case after *RFLP* analysis only three restriction fragments of 131, 123 and 83 bp were digested with *Hae III* enzyme. So all analysed individuals were monomorphic with homozygous genotype *mm* different by the present study with two heterozygous genotypes *Mm* and *mm*.

Table 2. Allelic frequency and genotypes of ***MSTN gene*** in Teleorman Black Head lambs

Animals	Genotypes %			Allele frequency	
	MM	***Mm***	***mm***	***M***	***m***
105	0	83.33	16.67	0.42	0.58

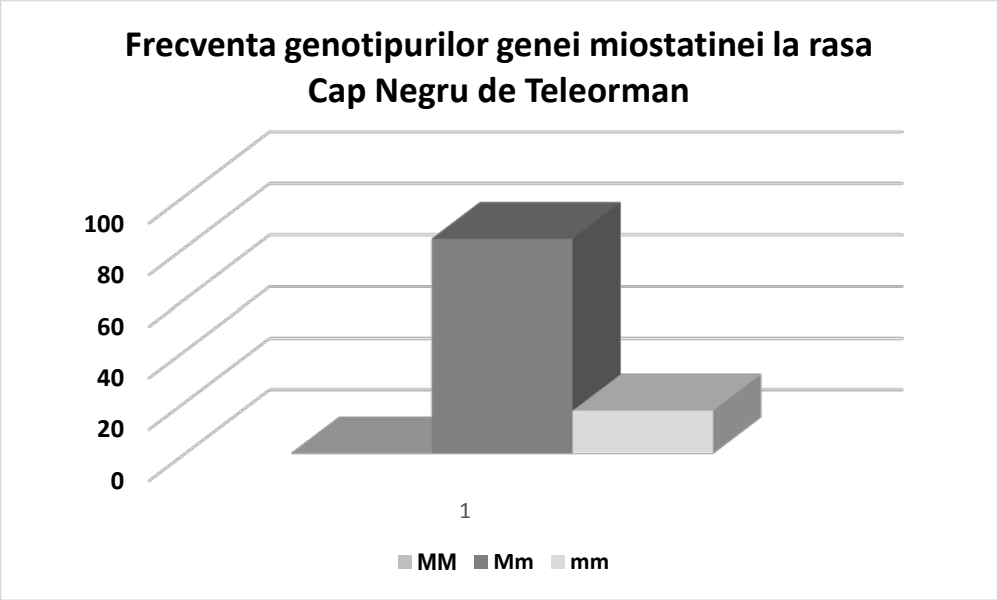

Figure 5. Genotypes frequency of myostatin gene in Teleorman Black Head lambs

Table 3. Observed and expected genotypes of ***MSTN*** gene in Teleorman Black Head lambs

Genotype	Observed	Expected
MM	0	19
Mm	90	53
mm	18	37
χ^2 at 1 DF and 5% significant degree	3.85	3.85 < 54.58*
χ^2 calculated	54.58	

*null hypothesis is denied

4. Conclusions

Two genotypes *mm* and *Mm* were identified in this study, homozygous genotype *mm* which does not carry *MM* mutation were in proportion of 16.67% and heterozygous genotype *Mm* were 83.33%. *M* allele frequency was 42% and for allele *m* was 58%.

MM individuals who carry this mutation were not identified in the analysed herd of the present study. This may be due to an empirical selection of this breed leading to the absence of this genotype. But there is the possibility to identify individuals who carry the important *MSTN mutation MM* because two alleles (*M and m)* were founded in this sheep population. That is why further investigations on a higher number of animals are necessary to discover this mutation correlated with meat quality in TBH sheep.

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