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MOLECULAR ANALYSIS OF OVINE CALPASTATIN (CAST) AND MYOSTATIN (MSTN) GENES IN LAMBS FROM THREE BULGARIAN SHEEP BREEDS

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ABSTRACT

The aim of present study was to investigate the polymorphism of calpastatin and myostatin genes in sixty lambs from three Bulgarian sheep breeds – 34 lambs from Synthetic Population Bulgarian Milk (SPBM), 13 lambs from Cooper-Red Shumen and 13 lambs from Karakachan. CAST and MSTN genes are considered as candidate genes for meat and growth traits. Blood samples were collected and genomic DNA was extracted using commercial purification kit. Genotypes were determined by PCR amplification followed by restriction fragment length polymorphism (RFLP) method. Based on results, calpastatin gene was found to be polymorphic in lambs from SPBM breed but in the other two breeds it was monomorphic. Myostatin gene was monomorphic in all tested animals. The allelic frequencies in CAST gene were 0,91 for allele M and 0,09 for N. Genotype frequencies were 0,82 and 0,18 for MM and MN, respectively. Observed and expected heterozygosity for this locus were 0,163 and 0,176, respectively. Established polymorphism of CAST gene in SPBM lambs could be used in future research for detection of possible association between genotype and meat tenderness.

Key words: sheep, calpastatin gene, myostatin gene, polymorphism, PCR-RFLP.

Introduction

Meat quality is an important economic indicator for farm animals. It is known that the quality of meat is determined by many factors such as breed, feeding, fasting, preslaughter handling, stunning, slaughter methods, chilling, storing conditions (Rosenvold and Andersen, 2003). In the last decade, more and more attention has been paid to the genetic basis of traits. With development of genetics and introduction of modern DNA technologies in farming, the production and quality of the obtained products is significantly increased and improved. Therefore it is mandatory to find potential genes associated with meat quality traits in different farm animals, including sheep, cattle, pigs, rabbits.

Genes of calpastatin and myostatin are two of the most studied genes associated with meat quality in sheep.

The calpastatin gene inhibits calpain activity and is important for regulation of most mortem meat tenderization, birth weight and weaning weight (Byun et al., 2008). The CAST gene is located at locus 5q15 (NC_040256.1) of the sheep genome (*Ovis aries L.*). It consists of 29 exons. First Palmer et al. (1998) identified the CAST gene in sheep by using PCR-RFLP method. They found polymorphism in the amplified fragment of 622 bp using *MspI* endonuclease enzyme, which digests the nucleotide sequence at specific site 5 '...C↓CGG...3'.

Myostatin is encoded by the MSTN gene growth differentiation factor 8 (GDF-8), which belongs to the family of transforming growth factors-β (Boman et al., 2009; Kaczor et al., 2019). Myostatin is an inhibitor of skeletal muscle growth and mutation in coding region leads to increased

muscling. The myostatin gene was first discovered in mice in 1997, as a negative regulator of skeletal muscle growth and development (McPherron et al., 1997). In sheep the MSTN gene is located at the end of the long arm of chromosome 2 of the sheep genome, at locus 2q32.2 (Miar et al., 2014). First Soufy et al., (2009) announced for the presence of three genotypes in exon 3 of MSTN gene in sheep.

The aim of present study was to identify genotype variants of calpastatin and myostatin genes in 60 lambs of three Bulgarian sheep breeds – Synthetic population Bulgarian milk, Cooper-Red Shumen and Karakachan, using PCR-RFLP method.

Materials and methods

Animals and DNA extraction

The experiment was carried out on 60 lambs from Synthetic Population Bulgarian Milk (SPBM), Cooper-Red Shumen (CRS) and Karakachan (KK) flocks of Agriculture Institute – Shumen (34 lambs of SPBM, 13 lambs of CRS and KK sheep breeds). The samples were collected from *v. jugularis* into vacuum tubes (3 ml) (Biosigma, Italy), containing anticoagulant EDTA.

Genomic DNA was extracted from the whole blood with Illustra Blood GenomicPrep DNA Purification Kit (GE Healthcare, UK) according to the manufacturer's instructions. The quality of the obtained about 10 – 50 ng DNA was determined using gel monitoring and spectrophotometry and samples were stored at -20ºC until the analysis was performed.

PCR amplifications

PCR amplifications were carried out in total volumes of 10 µl, containing 40 ng DNA template, 20 pM of each primer and 2× (1.5 mM $MgCl_2$) Red Taq DNA Polymerase Master mix (VWR, Int., Belgium). Two pairs of primers were used for amplifying each of the genes. For CAST locus was used primer set suggested by Palmer et al. (1998) and for MSTN locus by Dehnavi et al. (2012). The primer sequences are presented in Table 1.

Table 1: Investigated coli, region, primer sequencies and length of PCR fragments.

Locus	Region	Primer sequence	Length of PCR product
CAST	Exon 1C/1D and intron 1	F: 5'- TGG GGC CCA ATG ACG CCA TCG ATG -3' R: 5'- GGT GGA GCA GCA CTT CTG ATC ACC -3'	622 bp
MSTN	Exon 3	F: 5'- CCG GAG AGA CTT TGG GCT TGA -3' R: 5'- TCA TGA GCA CCC ACA GCG GTC -3'	337 bp

All PCR reactions were accomplished by QB-96 thermocycler (Quanta Biotech) under the following conditions presented in Table 2.

Table 2: PCR condition for amplification of the investigated regions of the CAST and MSTN genes

Locus	Primary denaturation	Cycle	Denaturation	Annealing	Elongation	Final elongation
CAST	95°C/5 min	30	95°C/30 sec	62°C/45 sec	72°C/1 min	72°C/10 min
MSTN	95°C/5 min		95°C/30 sec	58°C/45 sec	72°C/1 min	72°C/10 min

Restriction Fragment Length Polymorphism (RFLP) analysis

The genotypes of the analyzed individuals for the both genes were established using RFLP analysis. The digestion reactions were carried out in 10 µl final volume, containing 6 µl PCR product and 10 U/µl *MspI* restriction enzyme (Bioneer) for CAST gene and 10 U/µl *HaeIII* enzyme (Bioneer) for MSTN gene. PCR products were incubated at 37ºC for 15 h in thermoblock. The fragment sizes were separated and determined using GeneRuler™ Ladder, 50 bp (Fermentas) supplied with 1 ml 6xDNA Loading dye on 2,5% agarose gel and then visualized under UV light by documentation system (Canon UVDI, Major Science).

Statistical analysis

For statistical analysis was performed by means of allele and genotype frequencies, observed and expected heterozygosity, X^2 test and Hardy-Weinberg equilibrium.

Results and Discussion

After PCR amplification a 337 bp fragment for exon 3 of MSTN locus was obtained. *HaeIII* restriction enzyme was used to digest the PCR products. The enzyme cuts the *m* allele, but not M allele. Digestion of the m allele produced three fragments of 83, 123, and 131 bp (Figure 2). In this investigation all samples showed the *mm* genotype. As a result, all of them were monomorphic (Table 3, Figure 1).

Table 3: Number of alleles per locus (observed n_a and effective n_e), allele and genotype frequencies, average heterozygosity (observed Ho, expected He), chi-square for HWE and coefficient of inbreeding for MSTN gene.

Breeds	Allele number		Allele frequency		Genotype frequency			Heterozygosity	
	n_a	n_e	m	M	mm	mM	MM	H_o	H_e
SPBM	1,00	1,00	1,00	0,00	1,00	0,00	0,00	0,00	0,00
CRS	1,00	1,00	1,00	0,00	1,00	0,00	0,00	0,00	0,00
KK	1,00	1,00	1,00	0,00	1,00	0,00	0,00	0,00	0,00

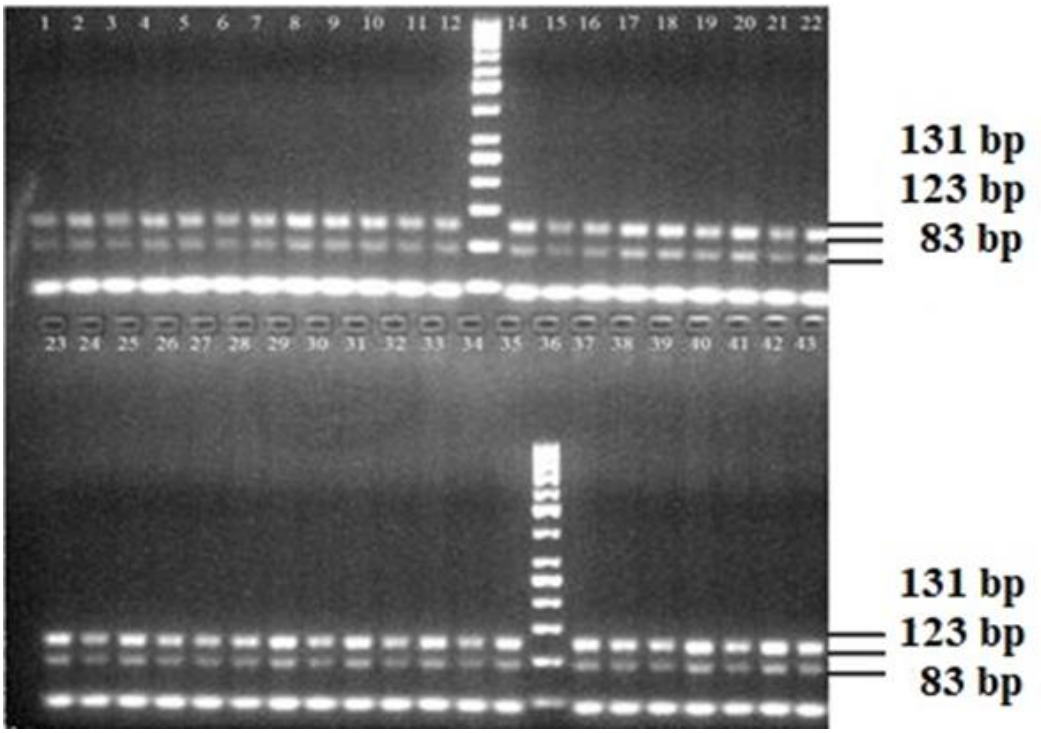

Figure 1: Restriction analysis of amplified products of MSTN gene with *HaeIII* restriction enzyme on 2.5% agarose gel by electrophoresis.

Results in this paper are in agreement with results reported in previous studies in three Bulgarian sheep breeds. Twenty-five adult animals (22 ewes and 3 rams) of Karakachan sheep breed, 32 rams of Northeast Bulgarian Merino and 121 sheep of Synthetic Population Bulgarian Milk were tested and all animals were carriers of the wild homozygous genotype – genotype mm (Georgieva et al., 2015; Dimitrova et al., 2016; Bozhilova-Sakova et al., 2016). Khederzadeh et al. (2016) presented the same results in 100 Zandi fat-tailed sheep in Iran. Azari et al. (2012) announced that exon 3 of MSTN gene was also monomorphic in 110 native Dalagh sheep. Elkorshi et al. (2013) studied the MSTN gene in 140 animals belonging to four Egyptian and two Saudi sheep breeds. All samples were analyzed by PCR-RFLP and only allele *m* and genotype *mm* were found.

Opposite to the results reported in this study Jamshidi et al. (2014) randomly collected 95 samples of Mehraban's sheep and announced for presence of the two alleles of MSTN gene with frequencies 0.97 and 0.03 for *m* and *M*, respectively. In Iran in Sanjabi sheep the researchers found genetic diversity in exon 3 of MSTN (Soufy et al., 2012). They also detected two alleles *m* and *M* with frequencies 0.97 and 0.03, respectively and all three possible genotypes – *mm*, *Mm* and *MM* with frequencies 0.97, 0.01 and 0.02, respectively. In the contrast in 105 Teleorman Black Head lambs in Romania in exon 3 of MSTN were identified genotypes *Mm* and *mm* with frequencies 0.83 and 0.17, respectively (Lazar et al., 2016). In this case the frequency of M allele was 0.42 and allele m was with frequency 0.58.

After PCR analysis of exon 1C/1D and intron 1 in CAST gene it was obtained a fragment of expected length of 622 bp. The MspI restriction enzyme digested the PCR products and two alleles

were detected. The enzyme cut the wild allele M, but does not cut the mutant allele N. Digestion of the allele M produced fragments of 336 and 286 bp.

In present study genetic polymorphism was found only in 34 lambs of Synthetic population Bulgarian sheep breed. The allele frequencies were 0.91 and 0.09 for M and N, respectively. The genotype frequencies were 0.82 and 0.18 for MM and MN. Genotype NN was not found. According to CAST gene the examined sheep population indicated non-significant deviation from HWE ($P<0.05$). Observed heterozygosity was 0.163 in the herd and expected heterozygosity was 0,176. F_{is} was 0,074 (Table 4, Figure 2).

Table 4: Number of alleles per locus (observed n_a and effective n_e), allele and genotype frequencies, average heterozygosity (observed H_o, expected H_e), chi-square for HWE and coefficient of inbreeding for CAST gene.

Breed	Allele number		Allele frequency		Genotype frequency			Heterozygosity		χ2	F_{is}
	n_a	n_e	M	N	MM	MN	NN	H_o	H_e		
SPBM	1,00	1,16	0,91	0,09	0,82	0,18	0,00	0,161	0,176	1,200[нз]	0,074
CRS	1,00	1,00	1,00	0,00	1,00	0,00	0,00	0,00	0,00		
KK	1,00	1,00	1,00	0,00	1,00	0,00	0,00	0,00	0,00		

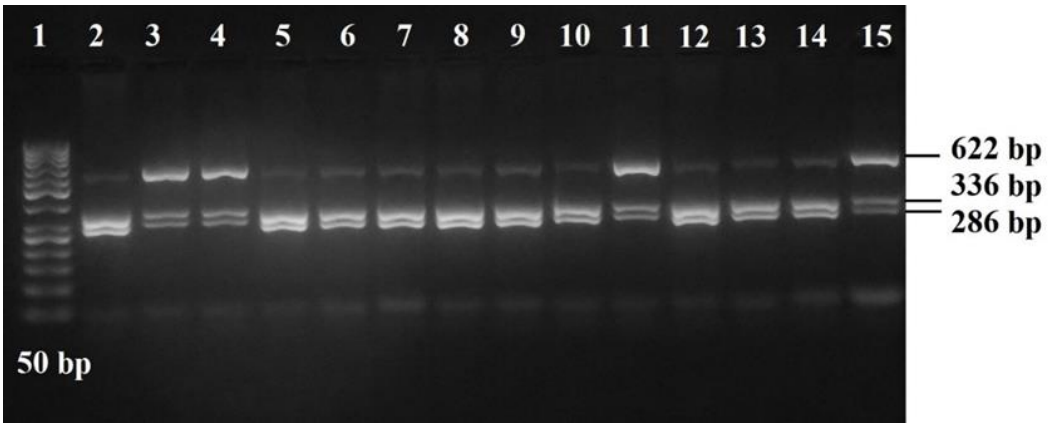

Figure 2: Restriction analysis of amplified products of CAST gene with MspI restriction enzyme on 2,5% agarose gel by electrophoresis in SPBM lambs.

The absence of polymorphism in tested region of CAST gene in lambs from Cooper-Red Shumen and Karakachan sheep breeds was in agreement with previous studies in local Bulgarian sheep breeds. Georgieva et al., (2015) also studied CAST gene in Cooper-Red Shumen and Karakachan sheep breeds but did not find genetic polymorphism. Bozhilova-Sakova and Dimitrova (2016) tested different population of Karakakchan sheep breed but genetic diversity was not found. Hristova et al., (2015) reported similar results in Local Karnobat sheep breed where only homozygous wild genotype MM was detected.

In agreement with the result found in lambs from SPBM in this study, Hristova et al., (2015) also reported for the presence of two alleles and two genotypes in Stara Zagora sheep breed. Allele M and genotype MM were with frequencies 0,97 and 0,94, respectively. Similar results were obtained by Gorlov et al. (2016) for the Salsk breed 0.78 for the MM genotype and 0.22 for the MN genotype. A study of Northeast Bulgarian Merino breeds showed these two genotypes too, but with a higher frequency of genotype MN – 0.53 and lower in genotype MM – 0.47 (Bozhilova-Sakova et al., 2020). In the contrast with the results in this study Kulikova et al, (2018) studied steppe type and mountain type of Tuvan short fat-tailed sheep breed. Authors identified all three possible genotype MM, MN and NN with frequencies 80.39%, 17.65% and 1.96% in steppe type and 77.00%, 22.00% and 1.00% in the mountain type, respectively.

Conclusion

Results showed polymorphism in CAST locus but MSTN locus was monomorphic in investigated sheep from Synthetic Population Bulgarian Milk breed. Two different genotypes (MM and MN) were showed in CAST locus in Synthetic population Bulgarian milk. In the other two breeds both loci were found to be monomorphic.

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