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Genotype frequencies in calpastatin (CAST) and callipyge (CLPG) genes in Northeast Bulgarian Merino sheep breed using PCR-RFLP method

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Abstract

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The purpose of the present work was to identify polymorphic allelic variants of ovine calpastatin and callipyge genes in Northeast Bulgarian Merino sheep breed. Blood samples were collected from 32 rams, reared in Agricultural Experimental Station – Targovishte. Genomic DNA was extracted using commercial kit for DNA purification from whole blood. After PCR amplification of CAST gene with specific primer set a fragment of 622 bp was obtained. CLPG gene also was amplified with specific primers and a fragment of 426 bp was obtained. Genotypes were determined by restriction fragment length polymorphism (RFLP) method with MspI restriction enzyme for CAST gene and BsmF1 restriction enzyme for CLPG gene. Calpastatin locus was found to be polymorphic, whereas in callipyge gene it was observed uniformity – the presence of genotype AA only. Genotype frequencies established in CAST gene were 0.47% and 0.53% for MM and MN, respectively, whereas genotype NN was not found.

Key words: sheep; Northeast Bulgarian Merino breed; CAST gene; CLPG gene; PCR-RFLP method

Introduction

Genetic diversity of modern domestic animal populations is very important for the improvement of the breeds, for their adaptation to different environmental conditions as well as obtaining of high-quality livestock products, meat particularly. The selection based on genetic markers for productivity is directed to working with animals with high genetic potential in terms of weight gain and meat quality. The development of skeletal muscles influences different enzymes, one of which is calpastatin (CAST). Calpastatin gene (CAST), which is localized on the 5q15 locus of chromosome 5 of sheep genome (*Ovis aries*), is polymorphic in many sheep breeds (Eftekhari-Shahroudi et al., 2006; Gabor et al., 2009; Ata & Cemal, 2012; Tohidi et al., 2013). In animals the formation of muscle mass requires a high activity of muscle calpastatin while after slaughtering lowest calpastatin activity contributes to obtaining high quality lean meat (Sazili et al., 2005). Calpastatin as an endogenous inhibitor of calpains that plays a central role in regulation of calpain activity in cell and is considered as one of the major modulators of protein metabolism. So it affects the proteolysis of myofibers and is responsible for post-mortem degradation of myofibrillar proteins. It regulates protein degradation, muscle growth as well as the rate and extent of processes in the meat after slaughtering – determines the speed of meat maturation. Data from the enzyme assay in sheep and cattle show inversely proportional correlation between levels of post-mortem muscle calpastatin with the best

meat tenderness (Palmer et al., 1999; Nowak, 2011; Sutikno et al., 2011; Bahrami & Ehsanizad, 2013). In general CAST gene affects economically important traits according to the requirements of the users such as tenderness and water content of the meat (Szkudlarek-Kowalczyk et al., 2011). This gene is related to both weight gain and carcass quality (Nassiry et al., 2006; Yilmaz et al., 2014). CAST gene is a potential candidate gene for control of livestock development (Ibrahim et al., 2015).

Callipyge (CLPG) is another gene affecting meat productivity in sheep, which revealed a non-Mendelian inheritance mode ("polar overdominance") – only paternal heterozygous animals develop mutant phenotype. CLPG is located near the telomeric end of sheep chromosome 18, within a cluster of imprinted genes. In this locus was found the best documented mutation in sheep that affects muscle growth and development, and which causes a postnatal muscle hypertrophy of the pelvic limbs and loin, but not in anterior skeletal muscles (Cockett et al., 1996; Vuocolo et al., 2007; Tellam et al., 2012).

In Bulgaria now are reared 34 sheep breeds including many indigenous breeds and others designed for specific purposes. Northeast Bulgarian Merino sheep is composite fine fleece breed created by crossing sheep of local breeds with Merino flaysh rams at the beginning and later with Askanian rams. In Shumen breed type (which is with high intensity of growth in young age) have been used also sires of Caucasian breed and less of Stavropol breed (Stancheva et al., 2015). This breed has significant impact on sheep production in the country. The animals are with good meat features. Sheep weight 65-70 kg, while rams 110-120 kg. Fertility is within 125 to 135%. The success of breeding strategies developed for different sheep breeds is directly related to the need for better knowledge of their inherited characteristics. Bulgarian sheep breeds are relatively poorly studied and it needs to be accumulated enough information about their genetic diversity for the purpose of successful breeding and selection.

The purpose of the present work was to identify polymorphic allelic variants of ovine calpastatin and callipyge genes in Northeast Bulgarian Merino sheep breed.

Materials and Methods

Blood samples were collected from thirty-two rams from Northeast Bulgarian Merino sheep breed, Shumen type, reared in Agricultural Experimental Station of Targovishte – Bulgaria. Approximately 3 mL of peripheral blood was collected from *v. jugularis* in vacuum tubes, containing EDTA. The laboratory analyses were carried out in the University of Forestry, in the Laboratory of Genetics of Agronomy Faculty. Genomic DNA was extracted by manual commercial kit for DNA purification from whole blood according to the manufacturer's instruction (QIAamp DNA Blood Mini Kit Qiagen). The DNA concentration was determined by spectrophotometer Biodrop.

PCR amplification reaction was carried out in total volume of 10 µl containing 4 µl DNA, 5 µl Red Taq Polymerase Master Mix (VWR) and 0.4 µl of each primer – forward and reverse (Bioneer). The primer sets are suggested by Palmer et al. (1998) for CAST and by Freking et al. (2002) for CLPG. The primer sequences are presented in Table 1.

After PCR amplification it was obtained a PCR product from CAST gene with length 622 bp. As a result of PCR reaction, it was amplified 426 bp fragment from CLPG gene. The PCR conditions for both genes are presented in Table 2.

Table 1. Primer sequences

Locus	Primer sequences	Fragment length (bp)
CAST	F: 5'-TGG GGC CCA ATG ACG CCA TCG ATG-3' R: 5'-GGT GGA GCA GCA CTT CTG ATC ACC-3'	622
CLPG	F: 5´-TGA AAA CGT GAA CCC AGA AGC- 3´ R: 5´-GTC CTA AAT AGG TCC TCT CG- 3´	426

Table 2. Conditions of PCR amplification

Steps	CAST		CLPG	
	Temperature	Time	Temperature	Time
Primary denaturation	95°C	5 min	95°C	4 min
Cycles	30 cycles		35 cycles	
Denaturation	95°C	30 s	95°C	20 s
Annealing	62°C	45 s	58°C	30 s
Elongation	72°C	1 min	72°C	1 min
Final extension	72°C	10 min	72°C	10 min
Store	10°C		10°C	

The digestion reaction for CAST was carried out in 10 µl final volumes, containing 6 µl PCR products and 4 µl restriction enzyme MspI with buffer (Bioneer) and was incubated at 37°C for 15 h. The PCR products from CLPG were digested separately in 10 µl final volume, containing 6 µl PCR product, 2.5 µl ddH_2O, 0.5 µl restriction enzyme BsmF1(New England BioLabs) and 1 µl enzyme buffer. The digestion reactions for CLPG were carried out at 65°C for 1 h in thermo block.

The fragment sizes were determined using GeneRuler Ladder, 50 bp (Sigma) supplied with 1 mL 6xDNA Loading dye. The obtained restriction products were tested on 2% agarose (Healthcare) gel, stained by 10 000 × RedGel TM Nucleic Acid Stain (Biotium) and visualized under UV light.

Results

The quality of extracted DNA from blood samples was tested on 1% agarose gel stained with GelRed (Figure 1).

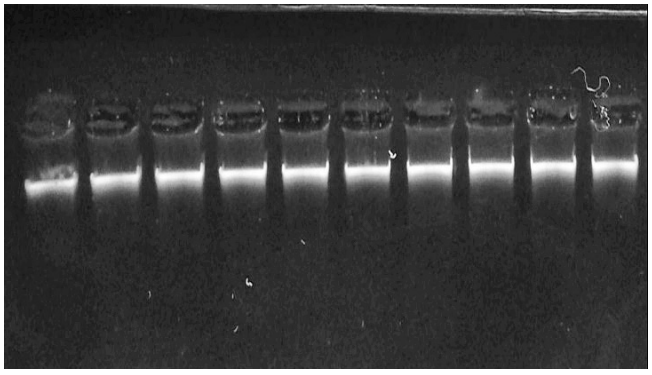

Fig. 1. Agarose gel electrophoresis for testing of extracted DNA samples

CAST locus

The application of PCR-RFLP analysis produced PCR fragments with length 622 bp in all tested samples. The resulting PCR product after treatment with restriction enzyme MspI gives two fragments with sizes 336 bp and 286 bp in the presence of allele M and in the presence of allele N – there is only one fragment with length of 622 bp. So it can be formed three genotypes – MM (two fragments with length of 336 bp and 286 bp), MN (three fragments – 622 bp, 336 bp and 286 bp) and NN (only one fragment with a length of 622 bp) (Palmer et al, 1998). In current study of rams from Northeast Bulgarian Merino sheep breed we observed presence of two alleles M and N and two genotypes MM and MN (Figure 2).

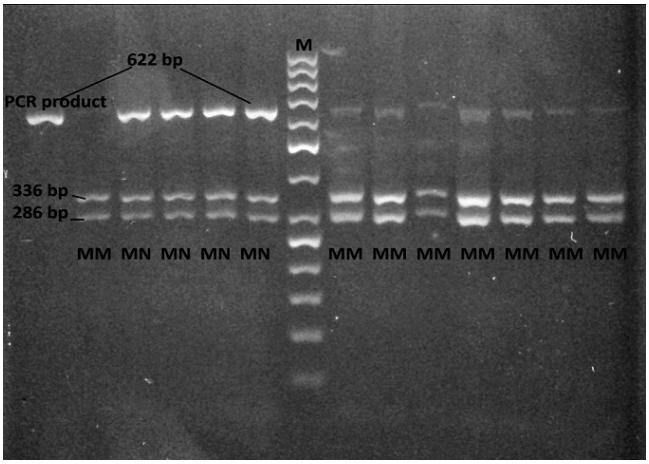

Fig. 2. PCR-product and restriction fragments of CAST gene of Northeast Bulgarian Merino rams, digested with enzyme MspI, observed on 2% agarose gel

The allelic frequencies were 0.73 and 0.27 for M and N, respectively (Table 3). The genotype frequencies were 0.47 for MM and 0.53 for MN. The genotype NN was not established in these animals from sheep breed Northeast Bulgarian Merino, although relatively non-low frequency of allele N – 0.27, presented in Table 3. The observed heterozygosity (0.531) is higher than expected heterozygosity (0.394).

CLPG locus

From CLPG gene was amplified a 426 bp fragment. The BsmF1 restriction enzyme digested the PCR products with formation of two alleles – wild allele A and mutant allele G. Allele A is characterized by the presence of three fragments with length – 278 bp, 117 bp and 31 bp. Allele G is presented by two fragments with length 395 bp and 31 bp. As a result it can formed three possible genotypes respectively – genotype AA (278 bp, 117 bp, 31 bp), genotype AG (395 bp, 278 bp, 117 bp, 31 bp) and genotype GG (395 bp, 31 bp) (Gabor et al., 2009). In this study was detected only genotype AA (Figure 3). Genotypes AG and GG were not determined in

Table 3. Allele and genotype frequencies of CAST locus

Locus	Allele frequency		Genotype frequency			Heterozygosity		$\varkappa^2$	Df	P value
CAST	M	N	MM	MN	NN	H_0	H_e	0.17	1	< 0.05
	0.73	0.27	0.47	0.53	0.00	0.531	0.394			

H_0 – observed heterozygosity, H_e – expected heterozygosity, $\varkappa^2$ – chi squarer, *Df* – degree of freedom
P-value < 0.05 nonsignificant

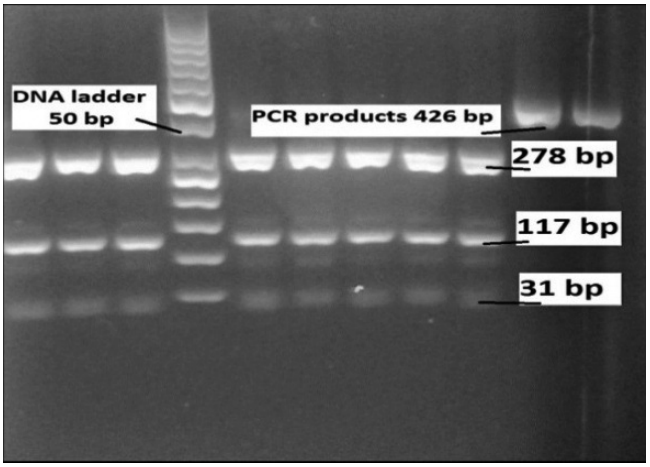

Fig. 3. PCR-product and restriction fragments of CLPG gene of Northeast Bulgarian Merino rams, digested with enzyme BsmF1, observed on 2% agarose gel

the herd. CLPG locus was found to be monomorphic in this population of 32 rams (Table 4).

Table 4. Allele and genotype frequencies of CLPG locus

Locus	Allele frequency		Genotype frequency			Heterozygosity	
CLPG	A	G	AA	AG	GG	H_0	H_e
	1.00	0.00	1.00	0.00	0.00	0.00	0.00

H_0 – observed heterozygosity, H_e – expected heterozygosity

Discussion

CAST locus

In present study of region 1C/1D of ovine CAST gene of rams from Northeast Bulgarian Merino sheep breed it was found polymorphism. Research of other Bulgarian breeds showed a significantly lower frequency of allele N in contrast of this one, or its complete absence. In study of other Bulgarian breed Synthetic Population Bulgarian Milk were observed frequency of allele N – 0.08, but also 0.01 frequency of genotype NN (Georgieva et al., 2015). In Bulgarian indigenous breed Stara Zagora (Hristova et al., 2015) reported frequency of allele N – 0.032 and absence of genotype NN. In another two autochthonous sheep breeds – Local Karnobat (Hristova et al., 2015) and Karakachan (Bozhilova-Sakova & Dimitrova, 2016) was detected only genotype MM, genotypes MN and NN were not determined. Observed frequency for allele N (0.27) in Northeast Bulgarian Merino rams is close to this determined for Karacul, Red Karaman and Kıvırcık breeds (Avanus, 2015), for Zel sheep (Gharahveysi et al., 2012), for Egyptian Osseimi, Barki and Rahmani breeds (Mahrous et al., 2015) and for Russian Salsk breed (Gorlov et al., 2016). The presence of all three genotypes (MM, MN and NN) reported in Soviet Merino sheep in Russia (Gorlov et al., 2016), in Iranian Kurakul sheep (Eftekhari – Shahroudi et al., 2006) and in Iranian indigenous sheepbreeds: Afshari, Ghezel, Makui, Arkhamerino, Sanjabi and Mehraban (Tohidi et al., 2013). Probably the observed heterozygosity in Northeast Bulgarian Merino rams (0.531) is higher than expected heterozygosity (0.394) due to the applied scheme of mating. Alakilli (2015) also observed higher heterozygosity in both investigated Saudi breeds Najdi and Harri as well as higher incidence of genotype MN and absence of genotype NN in the breed Harri as in studied Northeast Bulgarian Merino sheep breed.

CLPG locus

In investigated part of callipyge locus was observed monomorphism – the presence of genotype AA only, in investigated animals from Northeast Bulgarian Merino sheep breed. The heterozygous genotype AG and other homozygous genotype GG were not established. The same result was found in study of indigenous Karakachan breed in Bulgaria (Dimitrova & Bozhilova-Sakova, 2016). All investigated animals from India – from breeds Avicalin, Bharat Merino, Nellore, Chokla and Mapura (Kumar, 2012) and from Iran – Lori sheep breed (Nanekarani et al., 2014) are also monomophic for the same region of CLPG gene and obtain only wild genotype AA. Gabor et al. (2009) investigating 96 sheep originated from breeds and crosses Tsigai, Improved Valachian, Lacaune, East Friesian, and Tsigai sheep × Lacaune sheep and Alakilli (2015) in Saudi breeds Najdi and Harri found only the allele A and genotype AA.

Conclusion

In conclusion, we can summarize that the CLPG locus was monomorphic in Northeast Bulgarian Merino sheep breed with the presence of genotype AA and allele A only.

The study of the Northeast Bulgarian Merino rams showed genetic diversity of the calpastatin gene, which allowed the quality of the meat in this breed to be improved by selecting a suitable breeding program. For this purpose, however, it is necessary to study the relationship between the established polymorphism of CAST locus and the characteristics of the growth and quality of the meat in the breed.

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