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Molecular analysis of ovine calpastatin (CAST) and myostatin (MSTN) genes in Synthetic Population Bulgarian Milk sheep using PCR-RFLP

ABSTRACT

The aim of present study was to investigate the polymorphism of calpastatin and myostatin genes in sheep from Synthetic Population Bulgarian Milk (SPBM) breed because they are considered as candidate genes for meat and growth traits. Blood samples were collected from 121 sheep represented SPBM 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 but myostatin gene was monomorphic. The allelic frequencies in CAST gene were 92% for allele M and 8% for N. Genotype frequencies were 84%, 15% and 1% for MM, MN and NN in calpastatin locus, respectively. In this population, calpastatin locus did not show Hardy-Weinberg equilibrium ($P<0.05$). Observed heterozygosity for this locus was 0.148. Established polymorphism of CAST gene in SPBM sheep can be used in future research for detection of possible association between effective genotype and meat tenderness.

Key words: Synthetic Population Bulgarian Milk sheep breed (SPBM), genetic polymorphism, calpastatin (CAST) gene, myostatin (MSTN) gene, PCR-RFLP analysis

Introduction

Meat quality is one of the important economic traits in domestic animals. Determination of meat quality requires analysis and classification of fat content, composition, tenderness, water-holding capacity, color, oxidative stability, and uniformity. Meat quality is affected by several factors such as breed, genotype, feeding, fasting, preslaughter handling, stunning, slaughter methods, chilling, and storage conditions (Rosenvold & Andersen, 2003).

Finding of main genes responsible for meat quality will benefit the producers. In recent years, a lot of works have been performed in this field to find potential genes associated with meat quality traits in different farm animals, including sheep, cattle, pigs and poultry.

Calpains are intracellular calcium-activated cysteine proteases that have been implicated in a variety of physiological and pathological processes (Goll et al., 2003). In living muscle, calpains appear to be involved in degradation of myofibrillar proteins and they are believed to be the primary enzyme system responsible for post-mortem proteolysis that is the biochemical basis of meat tenderization (Koohmaraie, 1996). Calpastatin, an endogenous calpain specific inhibitor, inhibits the calpain activity in post-mortem tissue and thus regulates the rate and extent of post-mortem tenderization (Kocwin & Kuryl, 2003).

Myostatin (MSTN) or growth differentiation factor-8 (GDF-8) is a member of the mammalian growth transforming family (TGF-beta superfamily), which plays a role in the regulation of embryonic development and tissue homeostasis in adults (Sonstegard et al., 1998). Myostatin is an inhibitor of skeletal muscle growth and mutation in gene coding region leads to increased muscling.

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In Bulgaria, sheep meat is one of the major sources of animal protein and investigation for meat quality and related genes is important. Synthetic Population Bulgarian Milk (SPBM) sheep breed was registered as a breed in 2005 and now it is the most widespread in Bulgaria. The majority of the studies performed until now are related to the parameters defining the productivity or are aimed to identifying the effect of several factors (order of lactation, year of birth, linear differentiation, breeding age, type of udder and method of calculation) on the variability of phenotypic parameters of milk yield and biological fertility (Boikovski et al., 2003; 2005, 2006; Raicheva & Ivanova, 2010; 2011; Stancheva et al, 2013; 2014).

The aim of present study was to identify genotypes of calpastatin and myostatin genes in SPBM sheep using *PCR-RFLP* method in order to find effective alleles influencing meat quantity and quality traits in sheep.

Materials and Methods

Experimental animals and genomic DNA extraction

The present investigation was carried out on 121 adult sheep from Synthetic Population Bulgarian Milk flock of Agriculture Institute – Shumen (90 ewes and 31 rams). The samples were randomly collected from *v. jugularis* into vacuum tubes (3 ml) (Biosigma, Italy), containing 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. (1997) and for MSTN locus by Dehnavi et al. (2012). The primer sequences are presented in Table 1.

All PCR reactions were accomplished by GeneAmp thermocycler (Applied Biosistems, USA) under the following conditions presented in Table 2.

Restriction Fragment Length Polymorphism (RFLP) analysis

The genotypes of the analyzed individuals for the both genes were established using RFLP analysis. The digestion reaction was carried out in 10 µl final volume, containing 6 µl PCR product and 10 U/µl *MspI* restriction enzyme (Bioneer) for calpastatin gene and 10 U/µl *HaeIII* enzyme (Bioneer) for myostatin gene. PCR products were incubated at 37ºC for 15 h in thermostat. The fragment size was determined using GeneRuler™ Ladder, 50 bp (Fermentas) supplied with 1 ml 6xDNA Loading dye on 2% agarose gel and then visualized under UV light.

The obtained PCR product and restriction fragments were visualized with Electrophoresis Gel Imaging Analysis System (Bio-Imaging Sistems, Israel). GelCapture software was used for the image analysis of the bands. The restriction profiles are presented on the Fgures 1 and 2.

Table 1. *Locus, regions, primer sequence of PCR products of the CAST and MSTN genes*

Locus	Region	Primer sequence (5'→3')	Length of fragment (bp)
CAST	Exon 1C/1D and intron1	F: 5'- TGG GGC CCA ATG ACG CCA TCG ATG -3' R: 5'- GGT GGA GCA GCA CTT CTG ATC ACC -3'	622
MSTN	Exon 3	F: 5'- CCG GAG AGA CTT TGG GCT TGA -3' R: 5'- TCA TGA GCA CCC ACA GCG GTC -3'	337

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Table 2. *PCR condition for amplification of the polymorphic regions of the CAST and MSTN genes*

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

Statistical analysis

For statistical analysis were applied GENETIX software (Belkhir et al., 1996-2004), version 4.05 (05.05.2004) in order to estimate allele and genotype frequencies and ARLEQUIN software (Excoffier & Lischer, 2010), version 3.5.1.3 (09.2011) to estimate heterozygosity values and average genetic diversity and to check whether the examined population was in HWE.

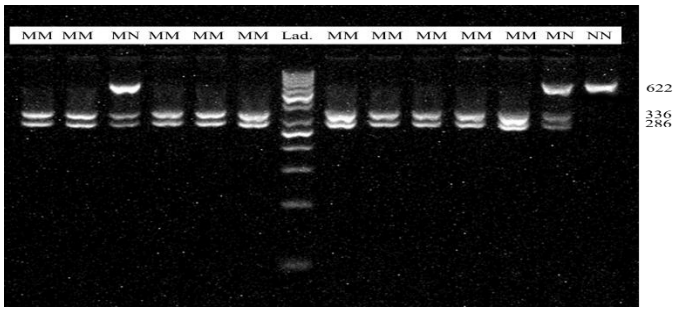

Figure 1. *Restriction analysis of amplified product of SPBM CAST gene with MspI restriction enzyme on 2% agarose gel electrophoresis.*

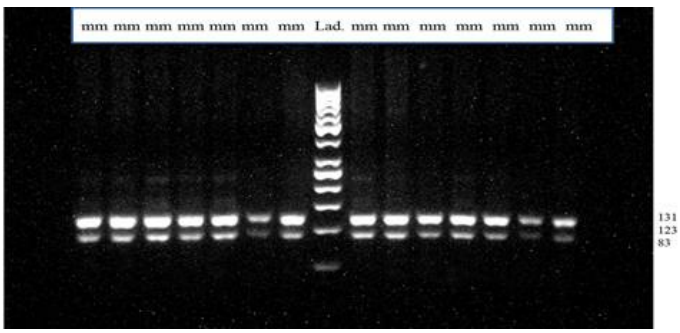

Figure 2. *Restriction analysis of amplified product of SPBM MSTN gene with HaeIII restriction enzyme on 2% agarose gel electrophoresis.*

Results and Discussion

Genetic polymorphism of CAST gene digested with MspI enzyme

A 622 bp fragment from CAST gene was amplified. The *MspI* restriction enzyme digested the PCR products and alleles of M and N were detected. The *MspI* digests the allele M, but not allele N. The *MspI* digestion of the allele M produced digestion fragments of 336 and 286 bp (Figure 1). The allelic frequencies were 0.92 and 0.08 for M and N, respectively. The genotype frequencies were 0.84, 0.15 and 0.01 for MM, MN and NN, respectively (Table 3). According to CAST gene the examined sheep population indicated non-significant deviation from HWE ($P<0.05$) (Table 3). Observed heterozygosity for this locus was 0.148 in the herd (Table 3). Expected heterozygosity (0.152) and expected heterozygosity calculated using Nei's method (0.151) were low. As a result genetic diversity with an average value of 0.076 was low.

Genetic polymorphism of MSTN gene digested with HaeIII enzyme

A 337 bp fragment for exon 3 of MSTN locus was amplifed. *HaeIII* restriction enzyme was used to digest the PCR products. The *HaeIII* digests the m allele, but not M allele. Digestion of the m allele produced three fragments of 83, 123, and 131 bp (Figure 2). All samples were digested by HaeIII enzyme and showed the mm genotype. As a result, all of them were monomorphic (Table 3).

Results showed polymorphism in CAST locus but MSTN locus was monomorphic in investigated sheep from Synthetic Population Bulgarian Milk breed. Three different genotypes (MM, MN and NN) were showed in CAST locus. Similar result for calpastatin locus was observed in Iranian Karakul sheep by Eftekhari Shahroudi et al. (2006).

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Table 3. *Number of alleles per locus (observed* n_a *and effecteve* n_e*), allele and genotype frequencies, average heterozygosity (observed* H_o*, expected* H_e*), average genetic diversity (**GDv**) and chi-square for HWE in the examined population for CAST and MSTN genes*

Locus	Allele number		Allele frequencies		Genotype frequencies			Heterozigosity			*GDv*	χ^2	*df***	*P****
	n_a	n_e	*M*	*N*	*MM*	*MN*	*NN*	H_o	H_e	H_{Nei*}				
CAST	2.00	1.18	0.92	0.08	0.84	0.15	0.01	0.148	0.152	0.151	0.076	0.02	2	0.98
			M	***m***	***M***	***Mm***	***mm***							
MSTN	1.00	1.00	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00				

** Expected heterozygosity computed using Nei's method (1973), ** degree of freedom (df) = 2, *** degree of probability (P) = 0.98*

A high degree of calpastatin polymorphism has also been reported in studies on Dorset Down hoggets, Dorset Down×Coopworth sheep, Corriedale rams and Angus bulls (Palmer et al., 1997; Chung et al., 1999). Palmer et al. (1997) detected three genotypes (MM, MN and NN) in unrelated Corriedale rams for this locus which was in agreement with the present results. Chung et al. (1999) observed AA, AB and BB genotypes for CASTI and CAST5 loci, and AA, BB, CC, AB, AC and BC genotypes for CAST10 locus in Angus bulls. In this population, CAST locus did not show Hardy-Weinberg equilibrium. This confirmed that factors leading to disequilibrium, especially selection, may affect genetic structure of population. Based on our results, the investigated population showed a good degree of genotypic variability for the CAST gene. This may be explained by the conservation and breeding strategies, which have been carried out.

In myostatin locus, all samples were digested by *HaeIII* enzyme and showed the mm genotype. As a result, all of them were monomorphic. Results from Dehnavi et al., (2012) in Zel sheep showed polymorphism in intron 2, but intron 1 and exon 3 were monomorphic. On the contrary, Soufy et al., (2009a, 2009b) observed polymorphism for exon 3 in Sanjabi sheep and native Kermanian cattle. Intron 1 was also monomorphic, and all samples showed the homozygotic genotype. On the other hand, intron 2 was polymorphic and three different genotypes were detected. Three different conformational patterns (AA, AB, and BB) were determined with frequencies of 73.5, 4, and 22.5%. The allelic frequencies for A and B were as 75.5 and 24.5%, respectively. Similar result was observed in Iranian Baluchi sheep (Ansary et al., 2008). This inconsistency may be ascribed to breed differences, population and sampling size, environmental factors, mating strategies, geographical position effect, and frequency distribution of genetic variants. Although myostatin locus was monomorphic in the Synthetic Population Bulgarian Milk sheep, results showed acceptable polymorphism for calpastatin locus, which may open interesting prospects for future selection programmes, especially using marker-assisted selection for improving weight gain and meat quality.

Conclusion

The goal of this study was to determine genetic polymorphism of CAST and MSTN genes in Synthetic Population Bulgarian Milk sheep. These results open up interesting prospects for future selection programmes, especially marker assisted selection. Results also confirmed that PCR-RFLP is appropriate tool for evaluating genetic variability.

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