Genetic variation of CAST gene in Local Karnobat and Karnobat merino sheep breeds

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Abstract. Karnobat sheep plays an important role in the development of sheep breeding in Southeastern region of Bulgaria. They are valuable source of genetic material. The aim of present experiment was to determine the allele variation of CAST gene in Local Karnobat and Karnobat Merino sheep breeds. A total of 60 blood samples were collected – 30 per breed. DNA was extracted and genotypes of all animals were identified by means of PCR-RFLP technique. The restriction reactions were accomplished by specific enzyme *MspI*. As expected both breeds were characterized with low level of genetic diversity due to the fact that mostly maintaining selection has been implemented. In Local Karnobat sheep breed was identified only one heterozygous individual from all 30. In Karnobat merino were identified allele M with frequency 0,97 and allele N with frequency 0,03. Genotypes MM and MN were revealed with frequencies 0,93 and 0,07, respectively. According to the statistical analysis both breeds were in HWE equilibrium.

1 Introduction

Sheep farming is an economically important industry for agriculture. Sheep breeding efficiency is directly affected by the quality and quantity of produced meat. Providing of lamb meat with higher quality is a key priority for this industry, because the main part of the income in the sheep farms, in particular in Bulgaria, is formed from the sale of lambs [1]. The quantity and quality of the obtained meat are formed under the influence of a complex of genes and environmental conditions. Improving the meat quality is the main objective of livestock production whereby the meat tenderness is one of the most important factors for measuring the quality of meat [2]. However, the quality of meat can hardly be improved by traditional selection, as the inheritance of meat quality is low [3] and the measuring of quality characteristics is difficult, expensive and possible only after slaughter. The application of new methods based on DNA technologies allows to make sheep breeding a modern, competitive and profitable industry [4]. In this regard, DNA methods based on the latest advances in molecular genetics are increasingly used to assess the genotype of breeding animals [5].

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The genes CAST (calpastatin), MSTN (myostatin), FABP3 (fatty acid binding protein 3), CLPG (calypig), DGAT1 (diacylglycerol acyltransferase 1) and LEP (leptin) were studied as candidate genes for meat productivity and meat quality in sheep [7]. One of the promising markers for growth intensity and meat quality in sheep is the calpastatin gene (CAST) [6, 7]. The CAST gene is located at locus 5q15 on chromosome 5 of the sheep genome (*Ovis aries L.*), it is composed of 29 exons separated by introns and has a total size of 89553 bp [8]. Genetic polymorphism has been identified in the following regions: exon 1, exon 6, exon 12, intron 12, and domains II to IV of the CAST gene [9, 10]. CAST inhibits calpain activity and this effects regulation of meat post mortem tenderness, birth weight, and growth rate to weaning. The calpastatin gene shows large and significant effects on birth weight and fat thickness [11]. A significant association between CAST genotypes and growth rate and final body weight was reported, with lambs of the MN genotype having a higher mean daily gain and final body weight than lambs of the MM genotype. The genotypes of the CAST gene have a significant effect on some components of the carcass and the quality parameters of the meat - the MN genotype shows a lower total bone mass and a higher meat to bone ratio compared to the MM genotype [12]. The presence of polymorphism in the locus of the calpastatin gene indicates that the quality of meat in the studied breeds of animals can be improved by applying an appropriate breeding program. Genetic polymorphisms, which are significantly associated with certain interesting traits, are very useful [13].

Different breeds of thin-tailed sheep are bred in Bulgaria - including local breeds and others intended for specific purposes such as milk, meat and wool. Many of the commercial Bulgarian sheep breeds are created based on local breeds. Such is the case with the composite Karnobat merino breed, in the creation of which were also used local Karnobat sheep characterized by high quality meat [14]. The establishment of genetic polymorphism in existing breeds of sheep is a major task, on the one hand in local breeds in order to preserve genetic resources, and on the other in cultivated animal breeds in order to their effective management and exploitation

The present study was aimed to investigate the genetic polymorphism of the CAST gene in 60 ewes of one local and one merino sheep breed – Local Karnobat and Karnobat merino.

2 Materials and Methods

The present experiment was conducted with a total of 60 ewes belonging to two sheep breeds from the Institute of Agriculture, Karnobat – 30 animals from Local Karnobat breed and 30 animals from composite Karnobat Merino breed. Blood samples were collected from the jugular vein in vacuum tubes containing EDTA as anticoagulant.

Genomic DNA was extracted from whole blood using a commercial purification kit according to the manufacturer's instruction (Illustra Blood Genomic Prep DNA Purification Kit, GE Healthcare). DNA concentration and purity were determined using spectrophotometer Biodrop and agarose electrophoresis on 1% agarose gel (Bioline) and TBE buffer (Jena Bioscience).

PCR reactions were performed in total volume of 10 µl containing 40 ng genomic DNA, 0.2 µl dd H_2O, 20 pM of each primer and 5 µl of ready-to-use 2×(1.5 mM $MgCl_2$) MyTaqTM HS Red Mix (Bioline). For the amplification of CAST gene was used primer set suggested by Palmer et al., [15]:

forward primer: 5'-TGG GGC CCA ATG ACG CCA TCG ATG-3'

reversed primer: 5'-GGT GGA GCA GCA CTT CTG ATC ACC-3'.

Amplification process was performed on thermal cycler QB-96 r (Quanta Biotech). The specific PCR conditions were shown on table 1.

Table 1. Specific PCR conditions for amplification of ovine CAST gene

Locus	Primary Denaturation	Cycles	Denaturation	Annealing	Elongation	Final elongation
CAST	94°C for 5 min	30	94°C for 30 s	62°C for 45 s	72°C for 1 min	72°C for 10 min

The genotypes of tested individuals were determined using RFLP method. The restriction reactions were carried out in 10 µl final volume containing 6 µl PCR product, 10 U/µl speed enzyme *MspI* (Jena Bioscience), buffer and ddH_2O. The incubation was performed in heat-block at 37^0C for 15-20 min. The fragment sizes were validated by agarose gel electrophoresis using 50 bp DNA Ladder (Thermo) on 2,5% agarose gel (Bioline) stained by 10000x RedGelTM Nucleic Acid Stain (Biotuim) and 1x TBE buffer (Jena Bioscience). The results were visualized under UV light.

All steps in the present experiment were carried out in Laboratory of Genetics, part of Agronomy Faculty in University of Forestry, Sofia, Bulgaria.

3 Results and Discussion

A 622 bp fragments from exon 1 and intron 1 of the CAST gene of the investigated animals were amplified by the described technique. All PCR products were subsequently subjected to enzymatic digestion with restriction endonuclease *MspI*. According to authors, this enzyme specifically cleaves the particular site with sequence 5' C↓CGG 3' and as a result produces two fragments with sizes of 336 bp and 286 bp, which define the allele M. The nucleotide sequences of 622 bp where the specific site 5'C↓CGG3' is absent remains uncut and determines the N allele. As a result, three genotypes can be observed - MM (with fragment size of 336 and 286 bp), MN (with 622, 336 and 286 bp) and NN (with 622 bp) [15].

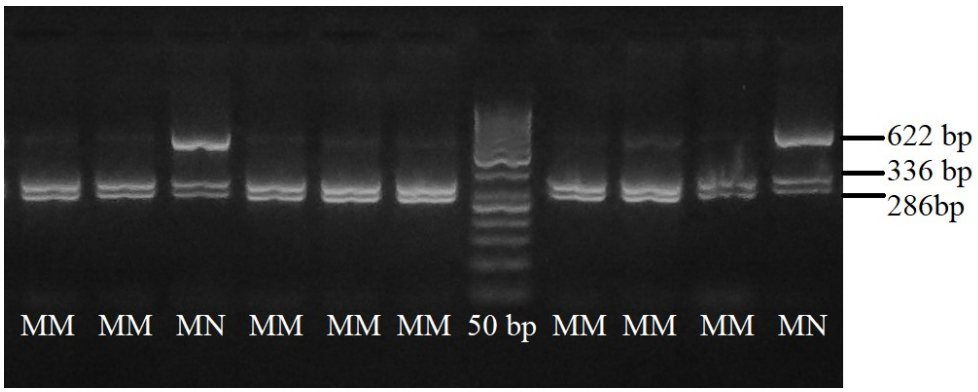

Fig. 1. DNA electrophoresis of CAST amplicons after digestion with *MspI* restriction enzyme: lane 7 - 50 bp DNA ladder, lanes 1, 2, 4 ,5, 6, 8, 9 and 10 - genotype MM, lanes 3 and 11 - MN genotype

In present experiment were determined two genotypes – homozygous genotype MM and heterozygous genotype MN (Figure 1). The most common allele in both breeds was the M allele. In the local Karnobat breed, the M allele was found with a frequency of 0.98 and the N allele with a frequency of 0.02. The Karnobat merino breed shows the following frequencies - of the allele M - 0.97 and of the N allele - 0.03 (Table 2). Both MM and MN

genotypes were identified in both studied breeds, while the NN genotype was not established. In the local Karnobat breed, we observed that the MM genotype occurred with a frequency of 0.97, and the heterozygous MN genotype - with a frequency of 0.03. The observed value of the MN genotype was slightly higher in the Karnobat merino breed - 0.07, while the frequency of the MM genotype was 0.93. According to the statistical analysis both breeds were in HWE equilibrium.

Table 2. Animal number, values of allele and genotype frequencies, average heterozygosity (observed Ho and expected He) and degree of probability for CAST gene in investigated breeds.

Breeds	n	Allele frequency		Genotype frequency			Ho	He	P*
		M	N	MM	MN	NN			
Local Karnobat	30	0,98	0,02	0,97	0,03	0,00	0,033	0,039	>0,05
Karnobat Merino	30	0,97	0,03	0,93	0,07	0,00	0,066	0,058	

*non-significant difference

The results in this study showed that the heterozygous genotype MN is present in the local Karnobat breed with very low frequency (0.02), while a previous study on the same sheep breed showed the absence of the heterozygous genotype [16]. Studies in other native Bulgarian breeds showed differences in the frequency of alleles of the CAST gene [17]. In the Cooper-Red Shumen and Karakachan breeds, a lack of diversity and only the M allele and the MM genotype were found [17, 18]. Two genotypes were identified in the Stara Zagora and Breznik breeds - MM and MN. In the Stara Zagora sheep the frequency of the mutant allele N is 0.03, of the genotype MM - 0.97 and of the heterozygous genotype MN - 0.03 [16], while in the Breznik breed there was a higher diversity - the frequency of the allele N was 0.12, of the genotype MM - 0.77 and of the heterozygous MN - 0.23 [17]. In studies of thin-tailed Turkish sheep breeds Kivircik, Imroz, and Karayaka, a null frequency of the NN genotype was found similar to the results in the present study, and in the Imroz breed the frequency of the M allele was 0.96 and of the heterozygous MN genotype was also close to this established by us - 0.07 [8].

The current study of Karnobat merino sheep showed low genetic diversity of the studied locus. The obtained results slightly, but statistically insignificant, exceeded the results obtained for the local Karnobat breed. In a previous study of animals of this breed, two alleles were found with a frequency of 0.94 for the M allele and 0.06 for the N allele and two genotypes - with a frequency of 0.89 for the MM genotype and 0.11 for the MN genotype [19]. Results with only two genotypes were reported in a study of other merino breeds – Caucasian, Northeast Bulgarian Merino, Volgograd, Salskaya and Stavropol [17, 20, 21, 22]. Three genotype variants were identified in the Askanian, Soviet merino and Altai Mountain breeds [17, 22, 23].

Many publications are devoted to the possible association of calpastatin polymorphism with important traits of meat productivity in different sheep breeds. Only two genotypes, MM and MN, were found in a study of Salsk sheep, and slaughter analyzes showed better meat productivity results related to the MN genotype [5]. In other molecular studies in sheep of the Volgograd breed also found the presence of two genotypes MM and MN. The results in control slaughter, showed that sheep with genotype MN/CAST superiored their peers with genotype MM/CAST by 3.7 kg in weight before slaughter [21]. Animals from Pakistani breed Kajli of heterozygous MN genotype grown faster compare to animals with MM genotype from birth to four months of age [24].

Identifying genes associated with productive traits would help improving the quality and diversity of production in the livestock industry by optimizing breeding programs. The

established polymorphism in the CAST gene shows that the study of this locus in Bulgarian sheep breeds must be studied in detail in order to be included in future breeding programs.

4 Conclusions

The results of this study showed low genetic diversity in the studied animals of both local Karnobat and Karnobat merino breeds. In both breeds, the presence of two allelic variants (M and N) and two genotypes (MM and MN) was found, with the M allele and the MM genotype predominance.

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