Polymorphism of CAST gene in five sheep breeds in Bulgaria

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Abstract. CAST gene is a candidate marker that influences the intensity of growth and meat quality. The aim of this study was to investigate the genetic variability of CAST gene in five Bulgarian sheep breeds – two merino, two local and one for milk. A total of 150 ewes, belonging to these breeds were investigated for polymorphisms of CAST gene by using PCR-RFLP method. A 622 bp fragment of *Ovis aries* CAST gene was amplified using PCR. After restriction with endonuclease *MspI* two alleles were observed in Ascanian, Caucasian, Breznik and Pleven Blackhead breeds. One allele (M) and one genotype (MM) were detected in Cooper-Red Shumen breed. The highest frequency of the allele N was established in the Ascanian merino breed (0.27) followed by Caucasian merino (0.13), Breznik (0.12) and Pleven Blackhead (0.07). Two genotypes - MM and MN, were observed in Caucasian, Breznik and Pleven Blackhead breeds. The three possible genotypes were found only in Ascanian merino ewes - MM, MN and NN. The lowest frequency of the homozygous genotype MM (0.50) and the highest frequency of the heterozygous genotype MN was established in the Ascanian merino breed (0.47). The obtained results confirm that the PCR-RFLP method can be used to identify different genotypic variation of CAST gene in Bulgarian sheep breeds. The established genetic diversity in the calpastatin gene indicates that, after further associative studies, this gene may be included in the breeding programs of certain sheep breeds.

1 Introduction

Meat quality and carcass weight are the traits, which are formed of a complex of genes and the environmental conditions. Molecular genetic studies have identified several genes that affect some quantitative characteristics, such as meat production [1, 2]. Marker assisted selection allows creation of individuals with specific DNA variations and hence improved productive traits which is much faster than conventional selection. The calpastatin gene (CAST) is considered as one of the promising markers for the intensity of growth and meat quality in sheep [3, 4, 5, 6]. The calpain-calpastatin system includes m-calpain (CAPN1),

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μ-calpain (CAPN2) and calpastatin and plays a key role in skeletal muscle development, participates in myoblast migration, protein metabolism, apoptosis, muscle growth and development, as well as fat deposition [7]. The calpastatin gene inhibits calpain activity and is important for regulating meat post mortem tenderness, birth weight, and growth rate to weaning [8]. In sheep, there is an inversely correlation between the level of muscle calpastatin after slaughter with the meat tenderness. CAST gene shows major and significant effects on birth weight and fat thickness [9].

The CAST gene is located at chromosome 5 locus 5q15 (NC_040256.1) of the sheep genome (Ovis aries L.) consisting of 29 exons. The first identification of the calpastatin gene in sheep was carried out by Palmer et al. [10] using the PCR-RFLP method. Polymorphism was detected in the amplified fragment of 622 bp from exon and intron 1 of CAST gene using MspI endonuclease, which digests the nucleotide sequence at the following specific site: 5 '...C↓CGG...3'. In this way have been identified two polymorphic variants, as a result from presence or absence of the point mutation (SNP) and changing of CC**G**G to CC**A**G [11, 12].

Establishing genetic polymorphism in existing sheep breeds is a major task, on the one hand for local breeds with the aim of conserving genetic resources, and on the other hand, for the efficient exploitation of existing breeds. Different sheep breeds there are in Bulgaria - including local breeds and others intended for specific purposes such as the production of milk, meat and wool. Seven breeds of sheep raised in Bulgaria have been studied so far [13 - 18] and four of them showed calpastatin gene polymorphism.

The purpose of the present study was to investigate the genetic polymorphism of the calpastatin gene in ewes of five Bulgarian breeds (two merino, two local and one for milk production) using PCR-RFLP.

2 Materials and Methods

The investigation was carried out in the Laboratory of Genetics of Agronomy Faculty, the University of Forestry.

2.1 Animals and blood collection

In present study were tested 150 ewes of five Bulgarian breeds -two merino (Ascanian and Caucasian), two local (Cooper-Red Shumen and Breznik,) and for milk production - Pleven Blackhead. Blood samples were collected from *v. jugularis* in vacuum tubes containing EDTA.

2.2 DNA extraction

Genomic DNA was extracted from whole blood using a commercial kit for DNA purification 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 1x TBE buffer (Jena Bioscience).

2.3 PCR amplifications

PCR amplification reactions were conducted in total volume 10 μl containing 40 ng genomic DNA, 0.2 μl dd H2O, 20 pM of each primer and 5 μl of ready-to-use 2×(1.5 mM MgCl2) MyTaqTM HS Red Mix (Bioline). For the amplification of CAST gene was used

the primers' pair suggested by Palmer et al., (1997) - forward primer: 5'-TGG GGC CCA ATG ACG CCA TCG ATG-3' and reversed primer: 5'-GGT GGA GCA GCA CTT CTG ATC ACC-3'. PCR reactions were carried out by using the QB-96 thermocycler (Quanta Biotech) under the following conditions: primary denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 45 s, elongation at 72°C for 1 min. The reaction was finished by final extension at 72°C for 10 min and was stored at 10°C forever.

2.4 Restriction Fragment Length Polymorphism

The genotypes of investigated animals were established using RFLP method. The restriction reactions were carried out in 10 µl final volume containing 6 µl PCR product, 10 U/µl endonuclease *MspI* (Bioneer), buffer and ddH_2O. The incubation process was performed in heat-block for 15h at 37ºC. The fragment sizes were determined by agarose gel electrophoresis using 50 bp DNA Ladder (Thermo) on 2% agarose gel (Bioline) stained by 10000x RedGel™ NucleicAcid Stain (Biotuim) and 1x TBE buffer (Jena Bioscience). The results were visualized under UV light.

3 Results and Discussion

The PCR results showed a 622 bp fragment of the CAST gene. Enzyme MspI produces two fragments of 336 bp and 286 bp at allele M, whereas at allele N, the PCR product remains uncut. After restriction with endonuclease MspI two alleles were observed in Ascanian, Caucasian, Breznik and Pleven Blackhead breeds and only one (M) in Cooper-Red Shumen. Only one genotype was detected in Cooper-Red Shumen – genotype MM (with two fragments - 286 bp and 336 bp). Two genotypes were observed in Caucasian, Breznik and Pleven Blackhead breeds - genotype MM (286 bp and 336 bp) and genotype MN (with three fragments - 286 bp, 336 bp and 622 bp). Three genotypes were found in Ascanian merino ewes MM, genotype MN and genotype NN (with one fragment – 622 bp) (Figure 1).

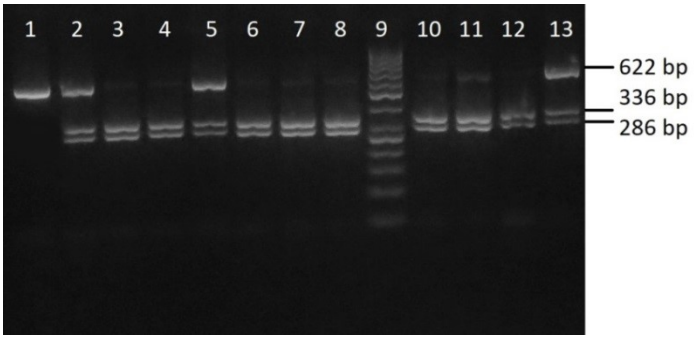

Fig. 1. DNA electrophoresis of CAST amplicons after digestion with *MspI* restriction enzyme: lane 9 - 50 bp DNA ladder, lane 1 – NN genotype, lanes 2, 5, 13 - MN genotype and lanes 3, 4, 6, 7, 8, 10, 11 and 12 – genotype MM.

The allele N was most common in the Ascanian merino breed (0.27) followed by Caucasian merino (0.13), Breznik (0.12) and Pleven Blackhead (0.07) (Table 1).

The highest frequency of the heterozygous genotype MN was at the Ascanian merino breed (0.47) followed by Caucasian merino (0.27), Brezhnik (0.23) and Pleven Blackhead

(0.13). The observed and the expected heterozygosity were calculated on the base of chi-square test using Hardy-Weinberg equilibrium. Populations Ascanian, Caucasian, Breznik and Pleven Blackhead were discovered to be in Hardy-Weinberg equilibrium for CAST locus. The coefficient of inbreeding varied from -0.182 in Ascanian breed, -0.177 in Caucasian and -0.111 in Pleven Blackhead sheep breeds to 0,104 in Breznik. It appears that the level of coefficient of inbreeding is very high in local Cooper-Red Shumen (Table 1).

Table 1. Number of animals, values of allele and genotype frequencies, average heterozygosity (observed Ho, expected He), coefficient of inbreeding and degree of probability for CAST gene in investigated breeds.

Breeds	n	Allele frequency		Genotype frequency			Ho	He	Inbreeding coefficient	p
		M	N	MM	MN	NN				
Ascanian	30	0.73	0.27	0.50	0.47	0.03	0.466	0.394	-0.182	P>0.01
Caucasian	30	0.87	0.13	0.73	0.27	0.00	0.266	0.226	-0.177	
Cooper-Red Shumen	30	1.00	0.00	1.00	0.00	0.00	0.000	0.000	1.000	
Pleven Blackhead	30	0.93	0.07	0.87	0.13	0.00	0.200	0.180	-0.111	
Breznik	30	0.88	0.12	0.77	0.23	0.00	0.233	0.211	-0.104	

The obtained results are in agreement with these reported earlier in other Bulgarian sheep breeds (Figures 2 and 3).

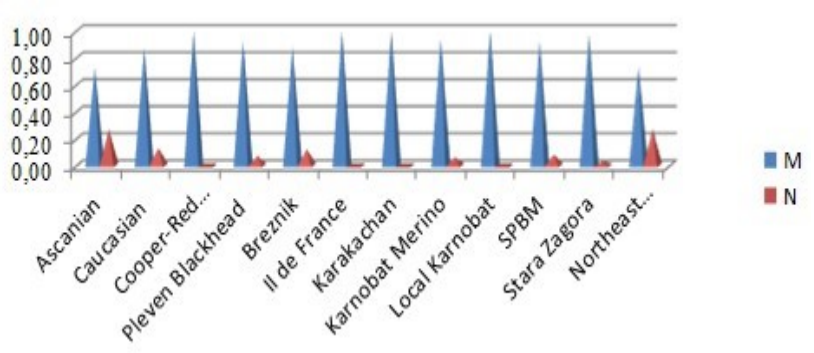

Fig. 2. Allele frequencies of CAST gene in investigated Bulgarian breeds.

The lack of diversity found in the local Cooper-Red Shumen breed coincides with that found for the other two aboriginal breeds - Karakachan [15] and local Karnobat [14], but similar results were also obtained for animals of the breed Il de France [16] (Figures 2 and 3).

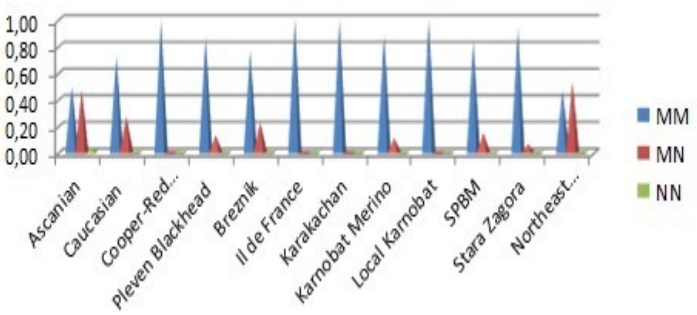

Fig. 3. Genotype frequencies of CAST gene in investigated Bulgarian breeds.

Animals of the Ascanian breed show the presence of the three possible genotypes MM, MN and NN at frequencies 0.50, 0.47 and 0.03, respectively. Three genotypes were identified in the study of the Synthetic population Bulgarian Milk sheep breed with frequencies 0.84, 0.15 and 0.01 for MM, MN and NN, respectively [13]. Similarly, three genotypes with frequencies of 0.82, 0.12 and 0.06 for MM, MN and NN, respectively, were identified at the Soviet merino breed [19], and at Turkish Kıvırcık crossbred ewes with frequencies of 0.82, 0.16 and 0.02, respectively [21]. At Tuvan short fat-tailed sheep breed three genotypes - MM, MN and NN, with similar frequency distribution have been identified (80.39%, 17.65% and 1.96% in steppe type and 77.00%, 22.00% and 1.00% in the mountain type, respectively) [5]. At the Prydniprovska meat sheep also have been reported the three genotypes, for MM the frequency is 0.77, for MN - 0.13 and for NN - 0.10 [22]. These three genotypes were also found in the CAST locus at the colombian creole sheep, the MM genotype was the most frequent (83.9%), followed by the other genotypes - MN (15.5%) and NN (6.0%) [23].

Many authors have conducted associative studies of the calpastatin gene with economically important traits in different sheep breeds. Pakistani sheep breeds Balkhi and Kajli with heterozygous (MN) genotype of this gene exhibited higher weight gain from birth to four months and eight months of age, respectively [24]. At Prydniprovska breed there is a tendency to increase the live weight of lambs carriers of N allele at 90 days of age (NN or MN) [23]. At sheep from Jordan breed Awasi with MN genotype have been reported higher final body weight, higher daily gain with a low conversion ratio and heavier longissimus muscle weight compared to MM animals, but the meat tenderness of the animals with MN genotype was lower than the ones with MM genotype [25]. West Siberian mutton breed sheep with the CAST MN genotype were also distinguished by the best average daily weight gain from birth to weaning by 6.9% and live weight by 20%, in relation to the sheep with genotype NN [26].

In this study, we identified satisfactory genetic diversity in the studied herds except in Cooper-Red Shumen. The highest genetic diversity was observed in both Merino breeds. The obtained results in genotyping allow tracing possible relationship with some characteristics of growth and meat quality in the future studies.

4 Conclusions

Improving the quantity and quality of sheep meat through traditional methods of selection is a costly and time-consuming process that can be accelerated by applying candidate genes with established genetic diversity. A suitable gene for this purpose in sheep is the calpastatin (CAST) gene. The obtained results confirm that the PCR-RFLP method can be used to identify different genotypic variation in Bulgarian sheep breeds. Genetic diversity

in CAST gene was identified in four of the studied breeds - Ascanian, Caucasian, Breznik and Pleven Blackhead. Monomorphism in this gene was observed only in Cooper-Red Shumen breed. Further studies are needed to establish associations between the different genotypes and their impact on the meat productivity characteristics of distinct sheep breeds and the possibilities for their inclusion in breeding programs.

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References

1. A.V. Deykin, M.I. Selionova, A.Y. Krivoruchko, D.V. Kovalenko, V.I. Truhachev, Vavilov J. of Genetics and Breeding, **20 (5)**, 576 (2016)
2. J. Knapik, K. Ropka-Molik, M. Pieszka, Ann. Anim. Sci., **17 (1)**, 23 (2017)
3. H. Chung, M. Davis, AJAVA, **7**, 641 (2012)
4. A. Ibrahim, I. Ismail, M.F. Shehata, A.R.El-Beltagy, J. Am. Sci., **11 (3)**, 106 (2015)
5. K. Kulikova, Y. Yuldashbaev, M. Petrovic, V. Caro Petrovic, D. RuzicMuslic, et al., Genetika, **50 (3)**, 885 (2018)
6. G. Gebreselassie, H. Berihulay, L. Jiang, Y. Ma, Animals, **10 (1)**, 33 (2020)
7. M. Greguła-Kania, T.M. Gruszecki, A. Junkuszew, E. Juszczuk-Kubiak, M. Florek, Meat Science, **154**, 69 (2019)
8. S. Byun, H. Zhou, R. Forrest, C. Frampton, J. Hickford,, Anim. Genet, **39**, 572 (2008)
9. E. Armstrong, G. Ciappesoni, W. Iriarte, C. Da Silva, F. Macedo, et al., Meat Science, **145**, 202 (2018)
10. B. Palmer, N. Roberts, J. Hickford, R. Bickerstaffe, J. Anim. Sci., **67**, 1499 (1998)
11. M. Greguła-Kania, Afr. J. Biotechnol., **10(61)**, 13082 (2011)
12. M. AhaniAzari, E. Dehnavi, S. Yousefi, L. Shahmohamadi, Slovak J. Anim. Sci., **45(1)**, 1 (2012)
13. S. Georgieva, D. Hristova, I. Dimitrova, N. Stancheva, M. Bozhilova-Sakova, J. BioSci. Biotech. **4(1)**, 95 (2015)
14. D. Hristova, S. Georgieva, S. Tanchev, AST **7(1)**, 36 (2015)
15. M. Bozhilova-Sakova, I. Dimitrova, Athens: ATINER'S Conference Paper Series (2016)
16. M. Bozhilova-Sakova, I. Dimitrova, In: Proceedings of Scientific Conference with International Participation “Animal Science - Challenges and Innovations”, 1(3), 332 (2017)
17. I. Dimitrova, M. Bozhilova-Sakova, M. Iliev, IOSR-JAVS, **10 (8)**, 61 (2017)
18. M. Bozhilova-Sakova, I. Dimitrova, T. Tzonev, N. Petrov, Bulg. J. Agric. Sci., **26(2**), 475 (2020)
19. I. Gorlov, N. Shirokova, A. Randelin, V. Voronkova, N. Mosolova, et al., Turkish J. Vet. Anim. Sci. **40(4)**, 399 (2016)
20. C. Sumantri, R. Diyono, A. Farajallah, I. Inounu, JITV, **13 (2)**, 117 (2008)

21. S. Kaplan, S. Atalay, Selcuk J. Agric. Food Sci., **31(3)**, 147 (2017)
22. I. Pomitun, V. Rossokha, Y. Boyko, O. Guzevatyi, M. Shpilka, et al., Agric. Sci. Pract., **6(2)**, 58 (2019)
23. D. Montes Vergara, C. Lenis Valencia, D. Hernandez Herrera, J. MVZ Cordoba, **24 (1)**, 7113 (2018)
24. S. Khan, M. Riaz, A. Ghaffar, M.Ullah, Pak J. Zool., **44(2)**, 377 (2012)
25. K. Jawasreh, R. Jadallah, A. Al-Amareen, A. Abdullah, A. Al-Qaisi, et al., Indian J. Anim. Sci., **87(5)**, 635 (2017)
26. A. Afanasyeva, V. Sarychev, G. Goncharenko, In: Proceedings of the International Scientific and Practical Conference “Digital agriculture - development strategy”, Advances in Intelligent Systems Research**,** 167, 116 (2019)