*IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)*
*e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 10, Issue 8 Ver. III (August 2017), PP 61-65*
*www.iosrjournals.org*

# Study of some genes associated with meat productivity in Karnobat Merino sheep breed using PCR-RFLP

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***Abstract:*** *The purpose of present study was the analysis of polymorphic variants of CAST, MSTN and CLPG genes associated with meat productivity in sheep. Karnobat Merino breed was established on the basis of local Karnobat sheep crossed with rams breed Kamvolmerino and Merinoflaysh and later Caucasus and Stavropol. Thirty five blood samples were collected from ewes of Karnobat Merino sheep breed. Genomic DNA was extracted and after PCR amplification with specific primers were obtained products with length 622 bp, 337 bp and 426 bp for CAST, MSTN and CLPG genes, respectively. The genotypes were determined using PCR-RFLP method with specific restriction endonucleases for each gene – MspI for CAST gene, HaeIII for MSTN gene and FaqI for CLPG gene. MSTN and CLPG genes were monomorphic. CAST gene was found to be polymorphic with allele frequencies: 0.94 for allele M and 0.06 for allele N and observed genotype frequencies: 0.89 for genotype MM and 0.11 for genotype MN. In this population the genotype NN was not established.*

***Keywords:*** *CAST gene, CLPG gene, Karnobat Merino sheep breed, MSTN gene, PCR-RFLP*

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Date of Submission: 20-07-2017 Date of acceptance: 08-09-2017

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## I. Introduction

In recent years the main role of sheep meat producers is to provide meat with good qualities such as tenderness, nice colorization, low consistence of water and etc. Therefore the selection is based on fast-growing animals with increased muscling.

There are three main genes affecting meat productivity in sheep and carcass quality which are mostly used in marker-assisted selection – calpastatin, myostatin and callipyge [3, 4, 5]. Calpastatin is an endogenous and specific inhibitor of calpains. Inhibiting the calpain activity in postmortem tissue CAST regulates the level of postmortem meat tenderization [6]. Myostatin is part of the mammalian growth transforming family (TGF-beta superfamily) [7]. Myostatin activity is related to inhibition of skeletal muscle growth. Mutations in MSTN locus could lead to increased muscling [5, 8]. Callipyge mutation causes muscle hypertrophy in pelvic limbs and loin when it's inherited from the father. This effect is called "polar overdominance" [9, 10].

Bulgarian fine fleece breeds are traditionally created by combining the specific characteristics of different breeds in order to increase productivity and genetic value and the resultant new breeds, populations and lines are classified as composite [11]. Bulgarian fine fleece sheep breeds have excellent meat productivity and adaptive ability, resistant to pests, insects and ticks and do not suffer from piroplasmosis. Karnobat Merino breed was established on the basis of local Karnobat sheep crossed with rams breed Kamvolmerino and Merinoflaysh and later Caucasus and Stavropol. In the Institute of Agriculture - Karnobat is kept the only herd of 170 pure-bred animals. Animals have a strong constitution and good exterior. Ewes have average live weight of about 55 kg, and rams - about 90 kg. Fertility is about 130% [12]. Nowadays because of the market needs it is important to analyze the allelic variation of the genes associated with increasing of the productivity and quality of sheep meat.

The aim of present study was to identify the allelic variants of three genes associated with meat productivity in sheep of Bulgarian breed Karnobat Merino.

## II. Materials And Methods

**Animals and blood collection**

In present study were tested 35 adult sheep of Bulgarian breed Karnobat Merino part of a larger population kept in the Institute of Agriculture, Karnobat. Blood samples were collected from *v.jugularis* in vacuum tubes containing EDTA.

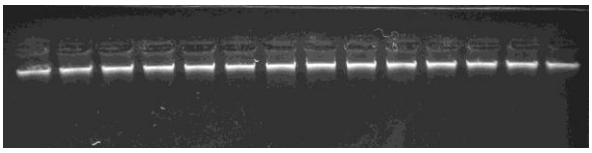

**Figure 1:** Genomic DNA tested on 1% agarose gel by agarose gel electrophoresis. All samples were with concentration approximately 10 ng/µl.

**DNA extraction**

Genomic DNA was extracted from whole blood with QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacture's instruction. DNA concentration and purity were determined using Biodrop and agarose electrophoresis on 1% agarose gel and 1x TBE buffer (Thermo) (Figure 1).

**Polymerase chain reaction (PCR)**

PCR amplification reactions were carried out in total volume 10 µl containing 4 µl genomic DNA, 5 µl Red Taq DNA polymerase Master Mix (VWR), 0.4 µl of each primer (Bioneer) and 0.2 µl ddH2O. For CAST gene was used primer sequence suggested by Palmer et al., (1997) [13], for MSTN locus by Dehnavi et al., (2012) [14] and for CLPG locus by Gabor et al., (2009) [15]. For each gene were used specific primer sequences showed in Table 1 and specific

**Table 1:** Locus, primer sequences and length of PCR fragments of the investigated genes

| Locus | Primer sequence (5'→3') | PCR fragment |
|---|---|---|
| **CAST** | F:5'-TGG GGC CCA ATG ACG CCA TCG ATG-3'<br>R:5'-GGT GGA GCA GCA CTT CTG ATC ACC-3" | 622 bp |
| **MSTN** | F: 5'-CCG GAG AGA CTT TGG GCT TGA-3'<br>R: 5'- TCA TGA GCA CCC ACA GCG GTC-3' | 337 bp |
| **CLPG** | F: 5'- TGA AAA CGT GAA CCC AGA AGC-3'<br>R: 5'- GTC CTA AAT AGG TCC TCT CG-3' | 426 bp |

PCR conditions showed in Table 2. All PCR reactions were accomplished by QB-96 Quanta Biotech thermocycler.

**Table 2:** PCR conditions

| Stages/Gene | CAST | | MSTN | | CLPG | |
|---|---|---|---|---|---|---|
| | **T°** | **Time** | **T°** | **Time** | **T°** | **Time** |
| Primary denaturation | 94 °C | 5 min | 94 °C | 5 min | 95 °C | 4 min |
| Cycles | 30 | | 30 | | 35 | |
| Denaturation | 94 °C | 30 s | 94 °C | 30 s | 94 °C | 20 s |
| Annealing | 62 °C | 45 s | 58 °C | 45 s | 58 °C | 30 s |
| Elongation | 72 °C | 1 min | 72 °C | 1 min | 72 °C | 1 min |
| Final elongation | 72 °C | 10 min | 72 °C | 10 min | 72 °C | 10 min |
| Store | 10 C° | | | | | |

**Restriction fragment length polymorphism analysis**

**Table 3:** Restriction conditions and restrictions enzymes of the investigated genes

| Locus | Endonuclease | Duration | Incubation °t |
|---|---|---|---|
| CAST | *MspI* (Bioneer) | 15 h | 37 °C |
| MSTN | *HaeIII* (Bioneer) | 15 h | 37 °C |
| CLPG | *FaqI* (Thermo) | 16 h | 37 °C |

The genotypes of investigated animals were established using RFLP method for the three genes. The restriction reactions were carried out in 10 µl final volume containing 6 µl PCR product, 0,5 µl specific endonuclease for each gene, buffer and ddH2O. The incubation process was performed at 37 °C in thermostat for all reactions. The restriction enzymes and conditions are shown in Table 3. The fragment sizes were determined by agarose gel electrophoresis using 50 bp DNA Ladder (Thermo) on 2% agarose gel stained by 10000x RedGelTM NucleicAcid Stain (Biotuim) and 1x TBE buffer. The results were visualized under UV light.

## III. Results And Discussion

**CAST locus**

The amplified region of CAST gene produced a 622 bp PCR fragment. After digestion with endonuclease *MspI* two alleles were observed allele *M* and allele *N* with frequencies 0.94 and 0.06 respectively. Enzyme *MspI* digests the allele *M* and as a result produces two fragments of 336 bp and 286 bp, but not the allele *N*. Only two genotypes were detected in this population – genotype *MM* (two fragments were observed –

286 bp and 336 bp) and genotype *MN* (three fragments were observed – 286 bp, 336 bp and 622 bp) (Figure 2) with frequencies 0.89 and 0.11, respectively (Table 4). Genotype *NN* was not found. Observed and the expected heterozygosity were calculated on base of chi-square test using Hardy-Weinberg equilibrium. This population was found to be in HWE for CAST locus.

These results are in agreement with results reported by other authors in different sheep breeds where the allele *M* was the most frequent [2, 13, 15, 17, 18, 19]. Hristova et al. [20] found similar results in another Bulgarian sheep breed. They investigated 96 animals from Local Karnobat and Stara Zagora sheep breeds and reported the presence of only two genotypes in Stara Zagora sheep breed with frequencies 0.97 and 0.03 for *MM* and *MN*, respectively. In previous our study in Synthetic population Bulgarian Milk sheep breed we detected the presence of all three possible genotypes of CAST gene with frequencies 0,84, 0,15 and 0,01 for *MM*, *MN* and *NN*, respectively.

Gorlov et al. [21] studied two breeds - Salsk sheep which have only 2 genotypes, *MM* and *MN*, with frequencies 0.78 and 0.22, respectively, and Soviet Merino sheep which shown 3 genotypes, *MM*, *MN*, and *NN*, with frequencies of 0.82, 0.12, and 0.06, respectively. Asadi et al. [22] also detected all three possible genotypes. Genotype frequencies in Dalagh sheep were 0.36, 0.38, 0.26 for the *MM*, *MN* and *NN* genotypes, respectively [23]. Santos et al. [24] investigating four Brazilian breeds Pantaneira, Ile de France, Suffolk and Brazilian Bergamacia have established the presence of all three genotypes in each of them. In Iran Tohidi et al. [25] found the presence of genotype *NN* with frequency more than 0,50 in Mehraban and Arcmerino sheep breeds. Sumantri et al. [26] studied animals from eight Indonesian sheep breeds and found extremely high level of genotype *NN* reach up to 1.00 in one of the breeds.

The obtained polymorphism in the CAST locus in Karnobat Merino population allows it to be used for improvement of meat quality in this breed through appropriate breeding programs.

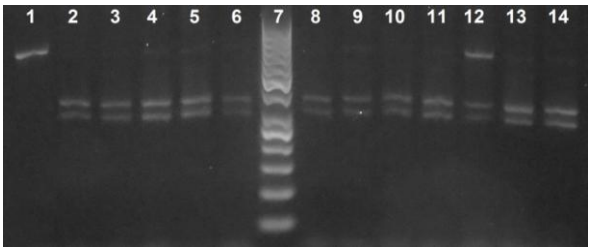


**Figure 2:** Restriction analysis of PCR product of CAST gene with *MspI* restriction enzyme on 2% agarose gel electrophoresis in Karnobat Merino sheep breed. 1 – PCR product 622 bp; 2, 3, 4, 5, 6, 8, 9, 10, 11, 13, 14 – homozygous genotype *MM;* 12 – heterozygous genotype *MN;* 7 – DNA Ladder 50 bp.

**MSTN locus**

After amplification of exon 3 of ovine MSTN gene a PCR fragment with size 337 bp was obtained. After digestion with specific enzyme *HaeIII* it was detected only allele *m* and only one possible genotype *mm* with frequency 1,00 *(*Table 4). *HaeIII* digests allele *m* and produces three fragments of 83 bp, 123 bp and 131 bp (Figure 3). Allele *M* and genotypes *Mm* and *MM* were not detected in this population. In the present study exon 3 of MSTN locus was found to be monomorphic.

Results in this paper are in agreement with results reported from different authors. In previous our studies in three Bulgarian sheep breeds we tasted 25 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 and we found similar results - all animals were carried only the genotype *mm* [5, 27, 28]. Ahani Azari et al. [23] reported that exon 3 of MSTN gene was also monomorphic in 110 native Dalagh sheep. Elkorshi et al. [29] 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. Khederzadeh et al. [30] obtained same results in 100 Zandi fat-tailed sheep in Iran.

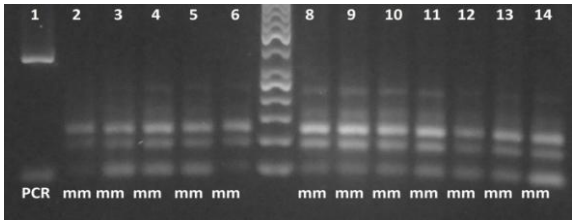


**Fig 3:** Restriction analysis of PCR products of MSTN gene with *HaeIII* restriction enzyme on 2% agarose gel electrophoresis in Karnobat Merino sheep breed. 1 – PCR product 337 bp; 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14 – homozygous genotype *mm;* 7 – DNA Ladder 50 bp.

Jamshidi et al. [31] randomly collected 95 DNA 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 was found diversity in exon 3 of MSTN [32]. They also detected the 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 was identified a high diversity in exon 3 of MSTN gene - two genotypes - Mm and mm with frequencies 0.83 and 0.17, respectively [33]. In this case M allele frequency was 0.42 and for allele m was 0.58.

**CLPG locus**

After PCR amplification reaction of ovine CLPG locus it was produced a fragment with length 426 bp. It was performed RFLP analysis with *FaqI* restriction enzyme which digests the PCR products and it was detected only the wild allele *A* - with three fragments of 278 bp, 117 bp and 31 bp. The mutant allele *G* (with two fragments 395 bp, 31 bp) was not observed in this study. As a result only one possible genotype was detected– genotype *AA* with frequency 1,00 (Table 4) (three fragments – 278 bp, 117 bp and 31 bp). The other two genotypes - genotype *AG* (four fragments – 395 bp, 278 bp, 117 bp, 31 bp) and genotype *GG* (two fragments – 395 bp, 31 bp) were not presented in this population. In this study CLPG locus was found to be monomorphic in Karnobat Merino sheep breed (Figure 4).

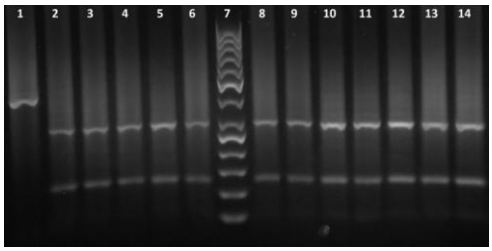


**Fig 4:** Restriction analysis of PCR product of CLPG gene with FaqI restriction enzyme on 2% agarose gel electrophoresis in Karnobat Merino sheep breed. 1 – PCR product of CLPG gene 426 bp; 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14 – homozygous genotype AA ;7 – DNA Ladder 50 bp

In previous our study in Bulgarian indigenous sheep breed Karakachan it was also observed only genotype AA with frequency 1,00 [34]. Same results were announced by Alakilli et al. [35] in two Saudi sheep breeds – Najdi and Harri. They were monomorphic for CLPG locus and only genotype AA was found. Gabor et al. [15] collected 96 samples from five different sheep breeds, kept in Slovakia. They observed only wild allele A in all tested animals. Same results were reported by Nanekerani et al. [4] in 124 Lori sheep. They all were monomorphic for CLPG locus.

In the contrast Jackson et al. [36] reported the presence of mutant allele G and the heterozygous genotype AG in crossbred of 15/16 Rambouillet and 1/16 Dorset rams.

**Table 4:** Frequencies of alleles and genotypes, observed (Ho) and expected (He) heterozygosity.

| Locus | n | Allele frequency | | Genotype frequency | | | | | | Heterozygosity | | $\chi^2$ | *p* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Observed | | | Expected | | | | | | |
| CAST | 35 | *M* | *N* | *MM* | *MN* | *NN* | *MM* | *MN* | *NN* | *He* | *Ho* | | |
| | | 0.94 | 0.06 | 0.89 | 0,11 | 0.00 | 0,88 | 0,11 | 0,01 | 0,112 | 0,114 | 0 | p>0.05 |
| MSTN | | *m* | *M* | *mm* | *Mm* | *MM* | *mm* | *Mm* | *MM* | *He* | *Ho* | | |
| | | 1.00 | 0.00 | 1.00 | 0.00 | 0.00 | 1.00 | 0.00 | 0.00 | 0.000 | 0.000 | 0 | |
| CLPG | | *A* | *G* | *AA* | *AG* | *GG* | *AA* | *AG* | *GG* | *He* | *Ho* | | |
| | | 1.00 | 0.00 | 1.00 | 0.00 | 0.00 | 1.00 | 0.00 | 0.00 | 0.000 | 0.000 | 0 | |

## IV. Conclusion

In present study it may be concluded that there is polymorphism in CAST locus of investigated animals from Karnobat Merino breed which could be used in future investigations referring to candidate genes for meat productivity in sheep. MSTN and CLPG loci were monomorphic in this population.

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Ivona Dimitrova. "Study of some genes associated with meat productivity in Karnobat Merino sheep breed using PCR-RFLP ." IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS), vol. 10, no. 8, 2017, pp. 61–65.